A COMPARATIVE STUDY FOR THE PRODUCTION OF RECOMBINANT
INTRACELLULAR GLUCOSE ISOMERASE BY ESCHERICHIA COLI AND
PICHIA PASTORIS

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A COMPARATIVE STUDY FOR THE PRODUCTION OF RECOMBINANT INTRACELLULAR GLUCOSE ISOMERASE BY <u>ESCHERICHIA COLI</u> AND <u>PICHIA PASTORIS</u>

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ABSTRACT

A COMPARATIVE STUDY FOR THE PRODUCTION OF RECOMBINANT INTRACELLULAR GLUCOSE ISOMERASE BY <u>ESCHERICHIA COLI</u> AND <u>PICHIA PASTORIS</u>

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In this M.Sc. study, intracellular thermostable glucose isomerase (EC 5.3.1.5) production capacities of two metabolically engineered microorganisms, *Escherichia coli* and *Pichia pastoris*, were investigated. In this context, to construct intracellular glucose isomerase (GI) producing recombinant *P. pastoris*, the cells were transfected with pPICZ-A expression vector containing GI coding gene (*xylA*) of *Thermus thermophilus*. After the confirmation of the transfection, effects of co-carbon source sorbitol were investigated. $C_{S0}=30$ g L^{-1} initial sorbitol concentration was found as optimal condition in terms of the cell growth and GI activity. The highest cell concentration and volumetric activity were attained as $C_X=$ 4.2 g L^{-1} (t=36h) and

A=792.9 U L^{-1} (t=24h), respectively; whereas the highest activity was obtained as A=545 U L^{-1} (24h) using methanol as the sole carbon source.

In the second part of the study, the research focused on GI production using *Escherichia coli* BL21 (DE3) pLysS carrying pRSETA::*xylA*$_{int}$. Laboratory scale air-filtered shake bioreactor experiments were designed to determine the effects of carbon sources on the cell growth of *E. coli*. The highest cell concentration was obtained in C_{M0}=30 g L^{-1} hydrolyzed molasses-based medium where the GI activity was A=2312 U L^{-1} being 3.17-fold higher than that of *P. pastoris*. Based on the revealed data, feeding strategy development experiments were carried out using *E. coli*. Effects of pulse and continuous feeding of molasses on GI production, the cell and by-product formations were investigated by four sets of pilot scale bioreactor experiments. The highest cell concentration was obtained as C_X=17.9 g L^{-1} at t=28h with a pre-determined exponential feeding calculated for the specific growth rate of μ_0=0.1 h^{-1}. In terms of GI activity, the most prospering strategy was PM-0.05. In this strategy, one molasses pulse given at t=7h and continuous feeding of molasses started at t=10h with a pre-determined exponential feeding rate of μ_0=0.05 h^{-1}. The highest volumetric GI activity was obtained as A=29050 U L^{-1} at t=26h of the bioprocess with $Y_{X/S}$=0.20 g g^{-1} overall specific cell yield on subsrate and 48.8 g total substrate consumption.

ÖZ

REKOMBİNANT HÜCRE İÇİ GLUKOZ İZOMERAZ ÜRETİMİ İÇİN ESCHERICHIA COLI VE PICHIA PASTORIS ÜRETİM POTANSİYELLERİNİN KIYASLANMASI

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Bu yüksek lisans tez çalışmasında, metabolik tasarımla değiştirilmiş rekombinant *Escherichia coli* ve *Pichia pastoris*'in ısıya dayanıklı hücre içi glukoz izomeraz (EC 5.3.1.5) üretim kapasitelerinin kıyaslanması amaçlanmıştır. Önce metabolik mühendislik teknikleri kullanılarak, thermofil *Thermus termophilus*'tan alınmış glukoz izomeraz genini taşıyan pPIZC-A::*xly*A plasmidi *P. pastoris* hücrelerine transfer edilmiştir. Transfeksiyon işleminin doğrulanmasının ardından, sadece metanol içeren ortamla rekombinant *P. pastoris*'in GI üretim kapasitesi araştırılmış ve A=545 U L^{-1} enzim aktivitesi elde edilmiştir. İkincil karbon kaynağı olarak sorbitolün GI enzimi üretimi ve hücre çoğalması üzerindeki etkisi araştırılmıştır. 30 g L^{-1} başlangıç sorbitol derişiminin hücre çoğalması ve enzim

aktivitesi açısından en uygun koşul olduğu bulunmuştur. En yüksek hücre derişimi ve hacimsel aktivite, sırasıyla, C_X=4.2 g L^{-1} (t=36st) ve A=792.9 U L^{-1} (t=24st) olarak elde edilmiştir.

İkinci alt-araştırma programında, pRSETA::$xylA_{int}$ taşıyan *Escherichia coli* BL21 (DE3) pLysS hücresiyle GI üretimi için laboratuvar ölçekli hava-filtreli çalkalamalı biyoreaktörlerde farklı karbon kaynaklarının hücre çoğalması üzerine etkisi araştırılmıştır. En yüksek hücre derişimi C_{M0}=30 g L^{-1} hidrolizlenmiş melas içeren ortam ile elde edilmiştir. Bu üretim koşulundaki en yüksek GI aktivitesi A= 2312 U L^{-1} olarak bulunmuştur, bu değer *P. pastoris* ile elde edilen aktivitenin 3.17-katıdır. GI üretimini arttırmak için, dört pilot ölçekli yarı-kesikli biyoreaktör deneyi *E. coli* konak hücresi kullanılarak tasarlanmıştır. Biyoreaktör ortamına anlık melas beslenmesi ve yarı-kesikli besleme stratejilerinin enzim aktivitesi, hücre derişimi, yan ürün oluşumu üzerindeki etkileri incelenmiştir. En yüksek hücre derişimi C_X=17.9 g L^{-1} olarak, μ_0=0.1 st^{-1} spesifik çoğalma hızına göre hesaplanmış üstel yarı-kesikli melas beslemesi ile t=28st'te elde edilmiştir. GI aktivitesi açısından en başarılı besleme stratejisinin t=7st'te verilen anlık melas beslenmesinin ardından t=10st'te μ=0.05 st^{-1} spesifik çoğalma hızına göre başlatılan yarı-kesikli melas beslemesini içeren PM-0.05 stratejisi olduğu görülmüştür. En yüksek hacimsel GI aktivitesi A=29050 U L^{-1} olarak t=26st'te elde edilmiştir. Substrata bağlı hücre verimi $Y_{X/S}$=0.20 g g^{-1} ve toplam substrat tüketimi 48.8 g olarak hesaplanmıştır.

Anahtar Kelimeler: Rekombinant hücre içi glukoz izomeraz, *E. coli, P. pastoris*, yarı-kesikli, besleme stratejisi.

To my family

ACKNOWLEDGEMENTS

Sena Yaman

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NOMENCLATURE

A	Volumetric activity	U L^{-1}
A_X	Specific activity	U g DCW^{-1}
C	Concentration	g L^{-1} or mol m^{-3}
DO	Dissolved oxygen	%
k	Reaction rate constant	
Km	Substrate concentration at which the rate of reaction is half of its maximum speed	M
N	Agitation rate	min^{-1}
Q	Volumetric flow rate	L h^{-1}
q	Specific formation or consumption rate	$\text{g g}^{-1}\text{ h}^{-1}$
r	Reaction rate	$\text{g L}^{-1}\text{ h}^{-1}$
t	Process time	h
T	Medium temperature	$^{\text{o}}\text{C}$
U	One unit of an enzyme	
V	Volume	L
Y	Yield	g g^{-1}
$\overline{Y}$	Overall yield	g g^{-1}

Greek Letters

ρ	Density	g L^{-1}
μ	Specific growth rate	h^{-1}
μ_0	Pre-determined specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Initial condition
O	Oxygen
p	Protein
S	Substrate/Sorbitol
X	Cell
G	Glucose
F	Fructose
M	Molasses

Abbreviations

AOX1	Alcohol oxidase 1
BAL	Benzaldehyde lyase
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
EC	Enzyme Commission
FLD1	Formaldehyde dehydrogenase 1
GAP	Glyceraldehyde-3-phosphate dehydrogenase
GRAS	Generally recognized/regarded as safe
GI	Glucose Isomerase
HFCS	High fructose corn syrup
HPLC	High performance liquid chromatography
ICL1	Isocitrate lyase 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LSLB	Low Salt Luria-Bertani

mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase A	Ribonuclease A
YPD	Yeast Extract Peptone Dextrose
XI	Xylose isomerase
xylA	Glucose/Xylose isomerase gene

CHAPTER 1

INTRODUCTION

Industrial biotechnology which is also known as white biotechnology is the application of modern tools and knowledge, such as genetic engineering, metabolic engineering, synthetic biology, systems biology, to production of low cost high value, sustainable and environmentally friendly products. Sources in industrial biotechnological processes are renewable substances, living organisms and enzymes prevalently (Tang and Zhao, 2009). Among the industrial biotechnological products, enzymes constitute the largest group. Enzymes are large protein molecules that are produced by living organisms and catalyze specific chemical reactions for sustainability of life (Smith, 1997). They are classified into six groups by Enzyme Commission (EC). Enzymes catalyzing oxidation/reduction reactions are called oxidoreductases (EC 1), transfer of a functional group are called transferases (EC 2), hydrolysis of a chemical bond are called hydrolases (EC 3), adding or removing groups non-hydrolytically are called lyases (EC 4), rearrangement of intramolecules are called isomerases (EC 5), the synthesis of new bonds to join two molecules are called ligases (EC 6).

In environmentally friendly industrial processes, enzymes have been used extensively. Sources and usage areas of industrial enzymes are presented in Table 1.1 (Arbige and Pitcher, 1989). Enzymes convert substrate into different substance(s) called product by forming complexes that reduce the activation energy necessary for the reaction. Enzymes are able to catalyze the reactions like inorganic catalysts. Since enzymatic reactions are specific and efficient, they have an important potential in industrial processes (Singhania *et al.*, 2010). Even though the chemical treatments

can be replaced by enzymatic reactions, the expense of the process can still be an obstacle when enzymatic processes are compared to inorganic or organic processes. At this point, modern biotechnological tools, e.g., recombinant DNA technology and large scale fermentation are used to overcome this issue by not only increasing the feasibility of the enzymatic processes but also improving the features of the enzymes such as ability to work at unnatural conditions. More than 90% of the enzymes in the industry are being produced with recombinant systems to increase the yield and productivity (Cherry *et al.*, 2003). According to the report published by BBC research, revenue of global industrial enzymes reached $3.6 billion in 2010 and is expected to reach $6 billion by 2016. Food and beverage enzymes comprising the biggest market share of industrial enzymes reached the revenue of $1.2 billion in 2010 and is expected to reach $2.1 billion by 2016 (Figure 1.1).

Microorganisms are more preferable sources than plant and animal cells due to having high proliferation rate and ease of the cultivation. Selecting suitable producing strain is a key point for industrial protein production. When an enzyme is produced intracellularly, it comes with the price of separation and purification steps after disruption of the cells. Therefore, selecting an extracellular enzyme producing microorganism is advantageous in terms of cost and simplicity of the process. In addition to that, GRAS (generally regarded as safe) status of the microorganism is an important selection criterion if the product is used in food process industry (Singhania *et al.*, 2010).

Microorganisms are divided into three groups according to the optimal growth temperature: psychrophiles (below 20°C), mesophiles (moderate temperatures), and thermophiles (high temperatures, above 55°C) (Brock, 1986). Due to having stability at high temperatures, thermophilic microorganisms and their enzymes have attracted the industry and taken part in many commercial applications (Demirijan *et al*., 2001). Operating the enzyme catalytic processes at high temperatures leads to higher reaction rates and yields as it engenders a decrease in viscosity, an increase in the solubility and diffusion coefficient of the components (Haki and Rakshit, 2003). Furthermore, as the operation temperature increases, contamination risk by other mesophiles decreases correspondingly and in case thermostable enzymes are

expressed by a mesophilic host, simple heat treatment can be used as a purification method (Vieille and Zeikus, 2001).

Table 1.1 Sources and usage areas of industrial enzymes (Arbige and Pitcher, 1989).

Enzyme	Source*	Usage Area
Bacterial α-amylase	*Bacillus subtilis* *Bacillus licheniformis*	Starch conversion
Fungal α-amylase	*Aspergillus oryzae*	Maltogenic saccharification
Amyloglucosidase	*Aspergillus niger var.*	Starch syrups, dextrose, foods
Pullulanase	*Klebsiella aerogenes*	Debranching starch
β-Glucanase	*Bacillus subtilis* *Penicillium emersonfi*	Brewing and food processing
Neutral protease	*Bacillus subtilis*	Brewing/flavoring
Alkaline protease	*Bacillus licheniformis*	Detergents
Neutral protease	*Aspergillus oryzae*	Baking
Cellulase	*Trichoderma spp.*	Cellulose hydrolysis
Invertase	*Yeast spp.*	Confectionery industry
Pectinase	*Aspergillus niger*	Fruit/wine processing
Anthocyanase	*Aspergillus niger*	Decolorizing grapes
Rennet	*Mucor spp*	Milk coagulant, dairy industry
Glucose isomerase	*Streptomyces spp.*	High fructose syrups
Lipase	*Mucorspp.* *Aspergillus*	Dairy industry, detergents, fat splitting
Lactase	*Saccharomyces lactis* *Kluyveromyces lactis*	Dairy industry
Hemicellulase	*Aspergillus niger*	Baking, fruits, gums
Glucose oxidase	*Aspergillus niger*	Analytical, food processing
Catalase	*Aspergillus niger*	Analytical, food processing

* Other organisms have also been used for the production of these enzymes.
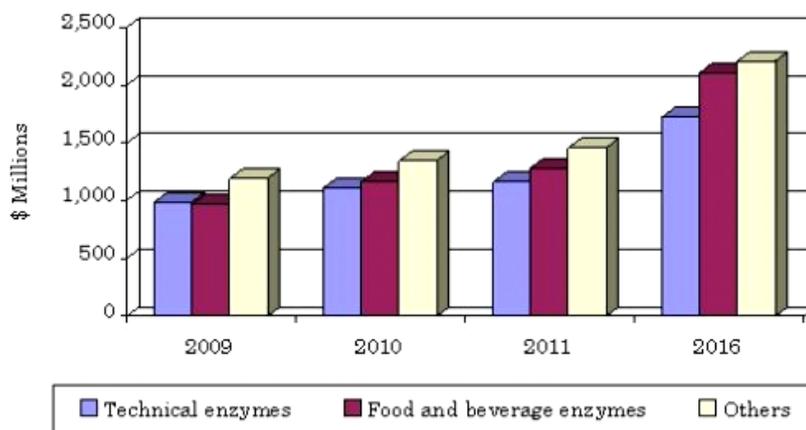
Figure 1.1 Global revenues of industrial enzyme market, 2009-2016, ($ millions).

D-glucose/xylose isomerase (D -xyloseketol isomerase; EC 5.3.1.5) is an enzyme, commonly known as glucose isomerase (GI), which catalyzes the reversible isomerization of D-glucose to D-fructose and D-xylose to D-xylulose, respectively. GI is mainly used for the production of high fructose corn syrup (HFCS) that depends on the conversion of glucose to fructose (Bhosale *et al.*, 1996). HFCS is an equilibrium mixture of glucose and fructose which is 1.7 times sweeter than glucose and 1.3 times sweeter than sucrose (Baker, 1976). Until 1976, the world had used sugar beet and sugar cane consisting of sucrose as the main sweetener. The concept of using GI for HFCS was first practiced in Japan. Then, it was followed by the United States due to the lack of sucrose supply after the Cuban revolution in 1958. Since then, it has been one of the most important enzymes in the industry (Bhosale *et al.,* 1996) 15 million tons/year HFCS is produced by using GI (Gavrilescu, 2005).

The other important usage area of GI is production of ethanol. Since bioconversion of xylose to xylulose is also catalyzed by GI, the enzyme offers to be used for bioethanol production by yeasts using renewable biomass. In this process biomass consisting of cellulose and hemicelluloses is converted to glucose and xylose. Then, this conversion is followed by ethanol fermentation performed by yeasts. Using GI, xylose, that is a considerable block in ethanol production metabolism, is converted to xylulose and fermentation of xylulose is performed by

such yeasts as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida tropicalis* at higher rates. (Chiang *et al.,* 1981-a; Chiang *et al.,* 1981b; Chan *et al.*, 1989; Gong, *et al.*, 1981; Schneider *et al.,* 1981; Wang *et al.,* 1980-a; Wang *et al.*, 1980-b).

Despite the fact that GI has been isolated from a broad range of bacteria (Chen, 1980), commercial GIs are obtained from mesophiles such as *Streptomyces* (Jargensen *et al.,* 1998), *Actinoplanes* (Gong *et al.*, 1980), *Flavobacterium* (Verhoff *et al.*, 1985). As the temperature increases, glucose: fructose equilibrium favors fructose (Bandish *et al.,* 2002). Therefore, using a thermostable GI obtained from a thermophile would be advantageous for HFCS production process where the production of fructose is the main concern.

Pichia pastoris is a methylotrophic yeast which is used for heterologous gene expression, extensively (Patrick *et al.*, 2005). It can express high levels of recombinant proteins under the control of alcohol oxidase promoter (*AOX*) by utilizing methanol as a carbon source (Tschopp *et al.,* 1987). The ability to secrete proteins at high levels and reach high cell densities in a simple defined media make *Pichia* expression system favorable for recombinant protein production (Cregg *et al*., 1993; Sberna *et al*., 1996). Owing to its ability to perform eukaryotic post-translational modifications and disulphide bridges, *P. pastoris* exhibits a considerable potential to be an alternative to bacterial expression systems (Cereghino *et al.*, 2002). More than 500 genes have been cloned and produced by *P. pastoris* (Macauley *et al.,* 2005). Typically *P. pastoris* fermentations are carried out in fed-batch mode by methanol inducible expression systems such as pPICZ-A (Invitrogen, San Diego, CA).

Escherichia coli is one of the most preferred microbial hosts for heterologous protein production from past to present (Terpe, 2006). The most likely reasons are rapid growth rate and protein expression, high product yield, simplicity of cultivation of *E. coli* (Swartz, 1996). Production of recombinant protein is performed in bioreactors generally using a defined medium in fed-batch operation mode with the purpose of reaching high cell density of *E.coli* (Shiloach and Fass 2005; Choi *et al*.,

2006; Shojaosadati *et al*., 2008). *E. coli* BL21 and K12 and their derivatives are commonly used for recombinant protein expression (Terpe, 2006).

In this M.Sc. study the aim is to compare thermostable glucose isomerase production capacity of a eukaryotic host *P. pastoris,* which has been extensively used for heterologous gene expression in the last decade and a prokaryotic host *E. coli,* which has been one of the most preferred bacterial expression systems. In the first part of this work, recombinant *P. pastoris* producing thermostable GI was constructed and thereafter, its intracellular recombinant GI production capacity was investigated. For this purpose, pPICZ-A plasmid containing thermostable GI gene, *xly*A, which had obtained from *Thermus thermophilus* (Ata, 2012), was integrated into *P. pastoris* strain X33 genome. Thereafter, laboratory scale air-filtered shake bioreactor experiments were performed with recombinant *P. pastoris* to investigate the effects of co-carbon source on the cell growth and GI activity. In the second part of this work, the effects of different carbon sources on cell growth of recombinant *E. coli* were examined in laboratory scale air-filtered shake bioreactors to determine the suitable carbon source for the production of GI. Afterwards, feeding strategies were developed for thermostable GI production using recombinant *E. coli* BL21 (DE3) pLysS strain carrying pRSETA::*xylA*$_{int}$ plasmid. The recombinant protein production medium containing hydrolyzed molasses was used initially in batch operation. Thereafter, effects of pulse and continuous feeding of molasses in semi-batch operation mode on GI activity, cell and organic acid concentrations were investigated in a pilot scale bioreactor.

CHAPTER 2

LITERATURE SURVEY

2.1 The Product: Glucose Isomerase

Glucose isomerase is an enzyme which catalyzes the isomerization reaction of D-glucose to D-fructose; D-xylose to D-xylulose, respectively (Figure 2.1). The first reaction occurs *in vitro*, while the second reaction occurs *in vivo* (Bhosale *et al.*, 1996). Examples of GI producing microorganisms and their yields are presented in Table 2.1. Since the enzyme performs isomerization reaction of glucose to fructose, it has been used for HFCS production (Antrim, *et al.*, 1979; Rehm and Reed, 1982).

HFCS production is accomplished in three steps. First, α-amylase is used to liquefy starch. Second, the starch is saccarified by amyloglucosidase and a debranching enzyme. Finally, using GI, glucose is converted to fructose. Product obtained at the end of these steps is a corn syrup with a considerable sweetening capacity higher than sucrose (Bhosale *et al.*, 1996).

HFCS production is the most dominant usage area of GI. 10^7 tons of HFCS is produced using immobilized GI per year. DuPont Industrial Biosciences (formerly Genencor) and Novozymes A/S are major immobilized glucose isomerase producers (DiCosimo *et al.*, 2013).
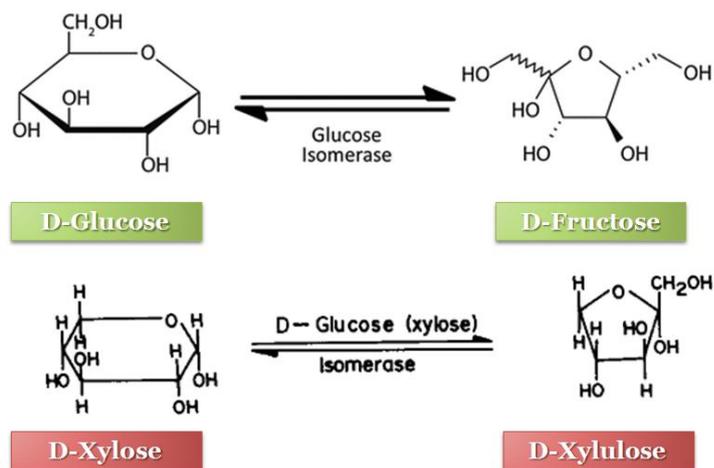
Figure 2.1 Enzymatic reactions catalyzed by GI.

GI is also used for ethanol production by enhancing the conversion of sugars of cellulosic biomass (Wang *et al.*, 1980-a; Chandrakant and Bisaria, 2000). When the hemicellulose of cellulosic biomass is depolymerizated, pentose sugars like D-xylose which cannot be utilized by common yeasts are produced (Wang *et al.*, 1980-b). In 1980, different forms of glucose isomerases were used to convert D-xylose to D-xylulose and fermentation of D-xylulose to ethanol was achieved using *Schizosaccharomyces pombe* and *Kluyveromyces lactis* (Wang *et al.*, 1980-a).

Characterization of GI was achieved by Marshall and Kooi for the first time in 1957 with D-glucose isomerizing enzyme obtained from *Pseudomonas hydrophila.* It was stated that the enzyme used D-xylose as a substrate but also was able to use D-glucose as an alternative with a 160 fold higher Km value. In the production process of GI, it was required to use media containing xylose and enhancement of the reaction was achieved with arsenate. Then, a patent on xylose isomerase (EC 5.3.1.5) that required neither arsenate nor NAD was filled by Tsumura and Sato in 1966. Since xylose is an expensive sugar to be used for industrial purposes, efforts were made and resulted with the identification of a xylose isomerase isolated from *Streptomyces* strain (YT-5) that grew in the media containing xylan from corn cobs (Takasaki, 1966). Today in commercial production of xylose isomerases,

microorganisms that do not require xylose are preferred (Wingard, 2012). GI production is typically performed by a submerged aerobic fermentation process lasting 2-3 days (Bhosale *et al.* 1996; Bhasin and Modi, 2012).

Thermodynamic equilibrium shifts towards fructose as the temperature increases. Industrial HFCS production is carried out with immobilized enzymes having the upper thermostability at 60°C. Production at this temperature gives the yield of 45% fructose in the syrup. This syrup is later fed with chromatographically purified fructose to obtain 55% fructose syrup which is used in food and soft drinks. Since high temperatures give high reaction yields, operating the isomerization reaction at 90-95°C using a thermostable GI could potentially eliminate expensive chromatographic methods. Therefore, efforts have been made to discover GIs that are stable at high temperatures, widely (Hartley, *et al.*, 2000). Equilibrium of isomerization of glucose to fructose with respect to temperature is represented in Figure 2.2 (Tewari, 1990).

Table 2.1 Glucose isomerase production by various microorganisms (Bhosale *et al.*, 1996).

Source	Enzyme Activity (U/liter)	Assay		References
		Temperature (^{o}C)	pH	
Actinoplanes missouriensis	2500-35200	75	7	(Anheuser-Busch Inc, 1974)
Bacillus licheniformis	10500	70	-	(Boguslawski and Rynski, 1982)
Streptomyces wedmorensis	560-2500	70	7.2	(duPreez, *et al.*, 1987)
Streptomyces olivochromogenes	4800-11400	60	7.5	Armbruster, *et al.*, 1973; Huang *et al.*, 1985)
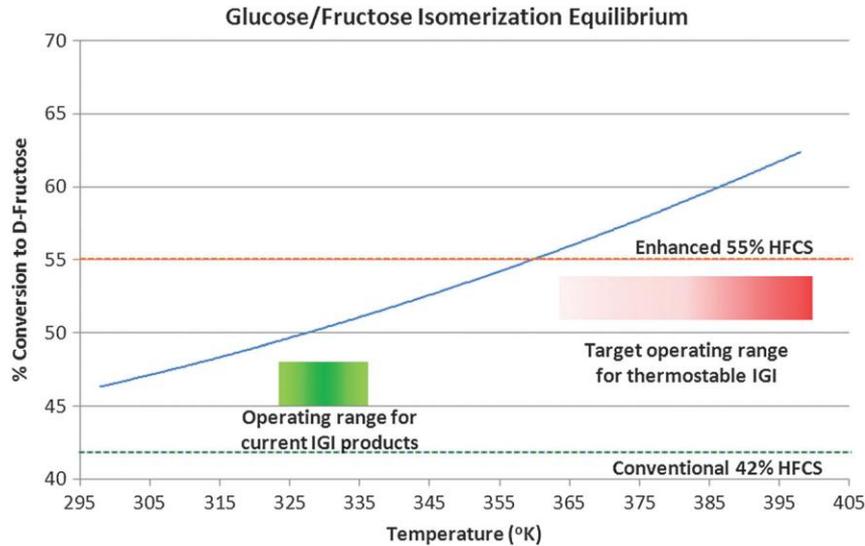
Figure 2.2 Conversion of D-glucose to D-fructose (Blue line represents the glucose/fructose equilibrium with respect to temperature) (Tewari, 1990).

2.1.1 Optimal Temperature and pH

GI has optimal temperature ranging from 60 to 80°C which increases in the presence of cobalt (Blacklow, *et al.* 1988). Enzymes obtained from thermophiles such as *Streptomyces* spp., *Bacillus* spp., *Actinoplanes missouriensis* and *Thermus thermosulfurogenes* exhibit higher stability at higher temperatures than mesophiles such as *Lactobacillus* and *Escherichia* spp. Optimal pH of GI ranges from 7.0-9.0 (Bhosale *et al.*, 1996).

2.1.2 Reaction Mechanism and Active Sites Studies

It was believed that catalytic mechanism of xylose isomerase involved histidine-directed general base catalysis (Carrell *et al.*, 1989). Subsequently, evaluating the X-ray crystallograpic studies of enzymes (Henrick *et al.*, 1989; Farber *et al.*, 1989; Collyer and Blow, 1990; Collyer *et al.*, 1990) obtained from *Arthrobacter* or *Streptomyces* and biochemical properties of thermophilic enzymes expressed by site directed mutagenized *xylA* gene which was obtained from

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Clostridium thermosulfurogenes, an alternative mechanism was suggested (Lee, *et al.*, 1990-a).

Xylose isomerase (XI) converts aldose to ketose proceeding in three steps (Figure 2.3) (Kovalevsky *et al*., 2010). The first step includes enzyme binding of substrate and ring opening of the substrate. The second step is isomerization of the substrate that is thought to proceed by metal ion-assisted hydride shift mechanism. Finally ring closure and release of the product occurs (Henrick *et al.*, 1989; Farber *et al.*, 1989; Collyer and Blow, 1990; Collyer *et al.*, 1990; Lee, *et al.*, 1990-a). Instead of ring opening reaction, the rate determining step is isomerization step (Lee, *et al.*, 1990-a). A suggested reaction mechanism of XI is presented in Figure 2.4 (Kovalevsky *et al*., 2010).

Since the difference between D-glucose and D-xylose is caused by an extra -CH_2OH group at the C-6 position in atomic configuration, this additional group is thought to be the reason for differences in substrate affinity between glucose and xylose (Mengshiao*et al.*, 1991).
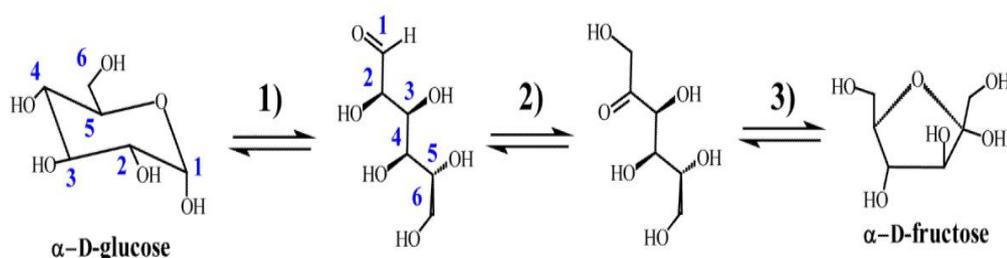
Figure 2.3 D-glucose-to-D-fructose interconversion reaction. (1) ring opening (2) isomerization (3) ring closure (Kovalevsky *et al*., 2010).
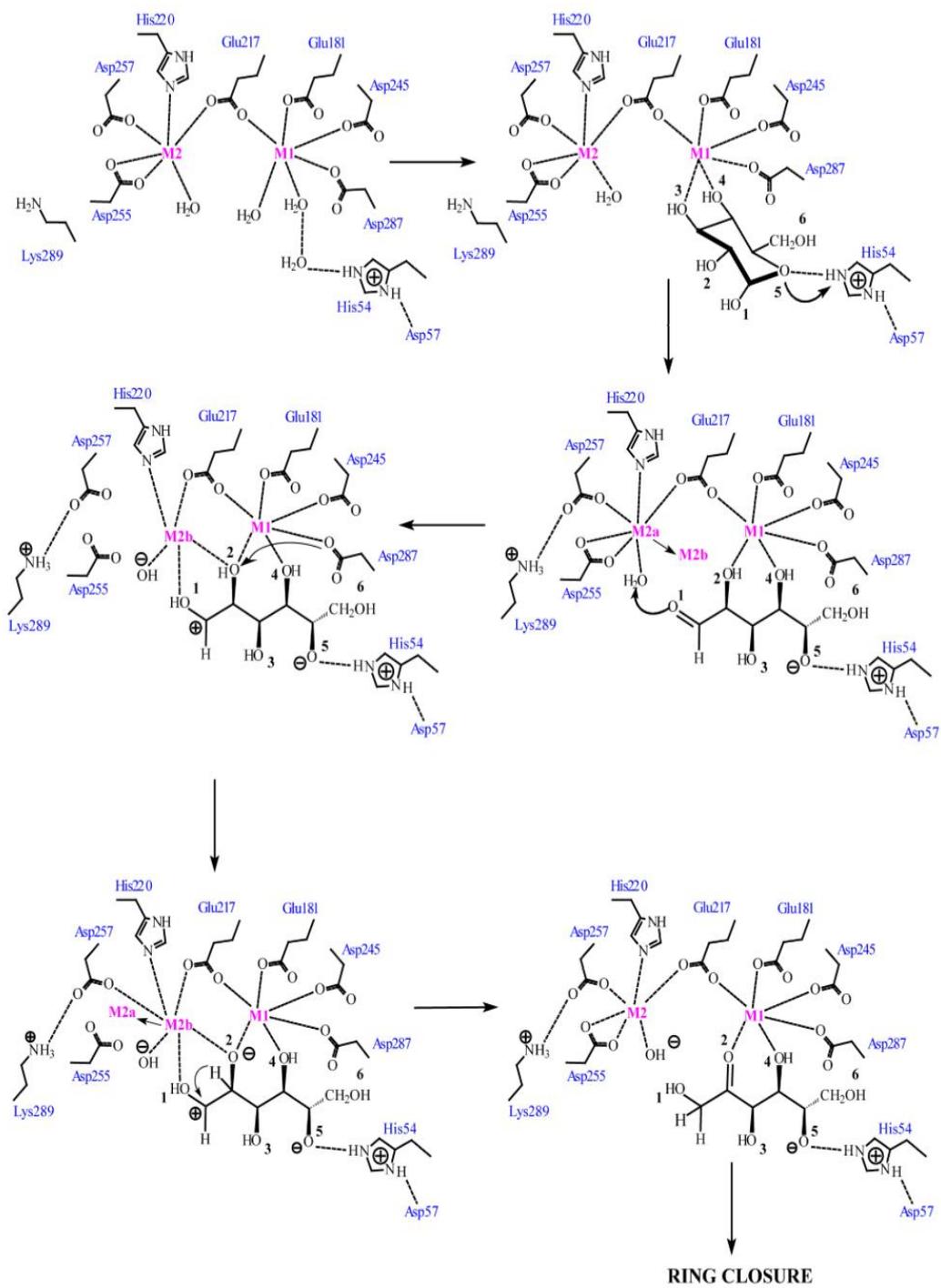
Figure 2.4 A suggested reaction mechanism of XI (Kovalevsky *et al*., 2010).

2.1.3 Structure of Glucose Isomerase

Depending on the microorganism, GIs vary from 44 to 191 kDa in molecular weight and have two or four subunits joined by non-covalent bonds. In some rare cases, trimer form of GI is produced as well (Chauthaiwale *et al.*, 1984). Glucose isomerase obtained from *Thermus thermopilus* is a homotetramer consisting of four identical subunits (monomer). A subunit of GI has 387 aminoacids at the molecular weight of 44000 Da (Dekker, 1991). Ghatge *et al.* (1994) stated that GIs having tetramer and dimer structure were active while monomer form of the enzyme was inactive. 3D structure of xylose isomerase obtained from *Thermus thermophilus* is presented in Figure 2.5 (BDP Access No: 1BXB).

There are two domains in the monomers of tetrameric structure. Each monomer consists ten β -strands, 16 α -helices, and five 3_{10}-helices. Domain I (residues 1 to 321) folds as (β/α) 8-barrel and Domain II (residues 322 to 387) contacts with Domain I (Chang *et al.*, 1999).

The enzyme is activated by homotetramer formation followed by two divalent ion binding to each active site of four monomer (Janis *et al.*, 2008).

Using X-ray crystallography together with neutron diffraction technique, metal binding sites of GI obtained from *Streptomyces rubiginosus* was examined by Kovalevsky *et al.* (2010). Requirement of two divalent metal cations of GI for full activity was stated and related sites were named according to their affinity to ions. The metal site which had affinity for Mg^{+2}, Mn^{+2}, Co^{+2}, Cd^{+2} and Pb^{+2} ions was called M1; while the metal site which showed a wider affinity for divalent ions was called M2. Metal binding active site of native GI is presented in Figure 2.6 (Kovalevsky *et al.*, 2010).
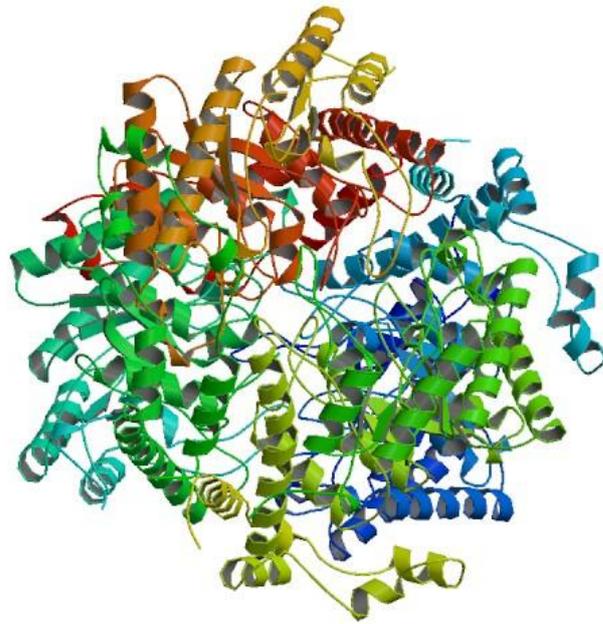
Figure 2.5 3D structure of xylose isomerase obtained from *Thermus thermophilus* (BDP Access No: 1BXB). Protein chains are colored from the N-terminal to the C-terminal using a rainbow (spectral) color gradient.
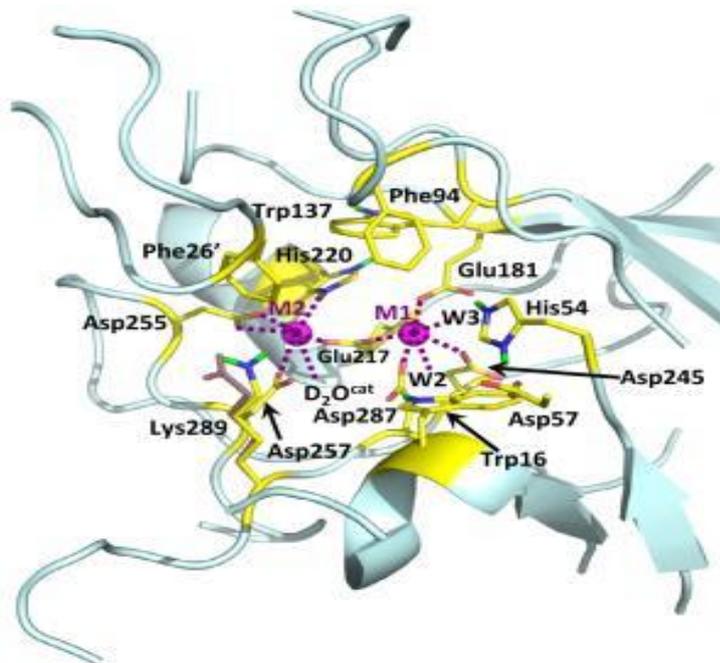
Figure 2.6 Metal binding active site of native GI (Kovalevsky *et al*., 2010).

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2.1.4 Metal Ion Requirements

Divalent cations such as Mg^{+2}, Co^{+2}, Mn^{+2} are responsible for activity and stability of glucose isomerase (Baker, 1975). Ion requirement in the fermentation medium depends on the particular species. While *Streptomyces* strain YT-5 (Takasaki and Tanabe, 1966) demands Co^{+2}, enzymes of *Bacillus coagulans* species demand Mg^{+2} or Mn^{+2} for enzyme production (Outtrup, 1974; Yoshimura *et al.*, 1966). GI requires both Mg^{+2} and Co^{+2} for the catalytic activity, essentially. Mg^{+2} is responsible for activity as Co^{+2} holds the enzyme in ordered conformation and provides the stabilization (Callens *et al.,* 1988; Callens *et al.,* 1986). Inhibitors of GI are Ag^+, Hg^{+2}, Zn^{+2}, Cu^{+2} and Ni^{+2}; xylitol, arabitol, sorbitol, mannitol.

2.1.5 Substrate Specifity

Maximum isomerization is achieved with substrates with hydroxyl groups at carbons 3 and 4 in the equatorial position, such as D-glucose and D-xylose (Chen, 1980). If the two are compared, GI shows higher affinity for xylose than glucose. Even though the substrate specifity of the enzyme depends on the type of microorganism, D-ribose, L-arabinose, L-rhamnose, D-allose, and 2-deoxyglucose can be converted by GI as well (Bhosale *et al.*, 1996).

2.1.6 Glucose Isomerase Production with Genetically Engineered Microorganisms

With the aim of overproducing glucose isomerase, genetic engineering techniques including the identification of GI gene and integration of the gene into a multicopy vector which enables the expression of the gene under an appropriate promoter have been applied. GI genes of diverse microorganisms have been used for this purpose. Both homologous and heterologous host cells have been utilized for cloning and expression of GI (Bhosale *et al.*, 1996).

The milestone of investigations of GI was the discovery and characterization of D-glucose isomerizing enzyme obtained from *Pseudomonas hydrophila* by Marshall and Kooi in 1957. GI gene was isolated for the first time in 1983 by *Ho et al.*

The GI gene of *E. coli* was integrated into *S. pombe* and expressed in the yeast by Chan *et al.*, (1989). Due to xylose isomerase activity of the yeast, xylose (10%, w/v) was utilized and ethanol (3%, w/v) was produced. Proteolytic degradation of the enzyme due to yeast proteases decreased the GI activity, therefore limited the fermentation of xylose.

Bacillus subtilis and *E. coli* were transformed by a plasmid, pMGL1 containing thermostable enzyme coding *xylA* gene which was obtained from *Clostridium thermosulfurogenes.* With the constitutive expression of GI gene, 5.3-fold higher enzyme activity (1.54 U mg^{-1}) for *B. subtilis* and 2.4-fold higher enzyme activity (0.46 U mg^{-1}) for *E. coli* were obtained compared with the activity of *C. thermosulfurogenes* (Lee *et al.*, 1990-b).

Dekker *et al.* (1991) cloned the GI gene of thermophilic microorganism *Thermus thermophilus* into *E. coli.* GI was produced under the control of *tac* promoter. 45-fold increase in the GI yield was obtained compared to the yield of *T. thermophilus*. Furthermore, with a simple heat treatment of crude extract at 85°C for 10 minutes, the GI was purified by 20-fold. Dekker *et al.* (1992) obtained 5600 U L^{-1} and 4300 U L^{-1} enzyme activity using *B. brevis* and *E. coli* expression systems, respectively.

In 1993, Wuxiang and Jeyaseelan cloned the thermostable enzyme coding gene of *Bacillus* sp. into *E. coli.* 12.8 U mg^{-1} specific GI activity at 85°C was obtained. The enzyme was purified via a single heat treatment process and the activity of the pure enzyme was reported as 49.02 U mg^{-1}.

In order to overproduce GI, *xylA* gene was inserted into the chromosome of *Streptomyces violaceoniger* using an integration vector (pTS55) providing a consitutive expression of GI. The gene of interest was integrated into the genome of *Streptomyces violaceoniger* in order to prevent plasmid loss during recombinant

protein expression. 7- fold higher GI activity was obtained without requirement of D-xylose induction (Bejar *et al.*, 1994).

Stevis and Ho (1995) performed high copy number cloning of isolated GI gene into the host microorganism, which was *E. coli* in this case. However, they did not meet with over-expression of GI due to the regulation of protein expression by the promoter presented in *E. coli*. Therefore, such strong promoters as *lac* or *tac* were combined with the gene of GI and the yield increase was 20-fold.

In 2005, bacterial *xylA* genes of *E. coli* or *Streptomyces coelicolor* were expressed in methylotrophic yeast *Hansenula polymorpha* under the control of glyceraldehyde-3-phosphate dehydrogenase gene promoter (*HpGAP*) by Voronovsky *et al*. Only approximately 18-23% of the GI activity of bacterial host cell was obtained by *H. polymorpha*.

In 2009 cDNA sequence of xylose isomerase was obtained from rumen fungus *Orpinomyces* by Madhavan *et al.* The gene was transformed into *Saccharomyces cerevisiae* for constitutive expression. The recombinant strain reached an ethanol yield of 0.39 g g^{-1} in xylose fermentation and 1.91 U mg^{-1} in specific enzyme activity.

xylA gene obtained from thermophile *Thermus thermophilus* was integrated into pRSETA plasmid by Angardi and Çalık (2013). The constructed plasmid was cloned into *E. coli* BL21 (DE3) pLysS and the highest thermostable enzyme activity was obtained as 16399 U L^{-1} in developed semi-batch bioreactor experiments using hydyrolyzed molasses-based media.

In M.Sc. study of Ata (2012) *xylA* gene obtained from thermophile *Thermus thermophilus* was integrated pPICZα-A plasmid and expressed under the control of *AOX1* promoter. The constructed plasmid was cloned in *P. pastoris* subsequently for extracellular production of thermostable glucose isomerase.

In M.Sc. study of Akdağ (2013), plasmid pRSETA::*xylA*$_{int}$ which was constructed by Angardi and Çalık (2013) was transformed into sucrose utilizing *E.*

coli W and the highest thermostable enzyme activity was reported as 35264.5 U L^{-1} in developed semi-batch bioreactor experiments using unhydrolyzed molasses-based medium.

Relatively high enzyme activity for extracellular GI was reported as 41000 U L^{-1} by Pandidurai *et al.* (2011) produced with a natural microrganism, *Enterobacter agglomerans*, when xylose was used as carbon, peptone was used as nitrogen source.

2.2 Host Microorganisms

Most bioproducts are produced by recombinant DNA technology today, in order to fulfill the requirements of the industry. For the expression of recombinant proteins, the desired DNA that encodes protein of interest is inserted to a host microorganism. Host microorganisms for recombinant protein production can be bacteria, yeasts, molds, mammalian, plant and insect cells. Quality of the produced protein, speed, economy and yield of the process are important points to be considered while choosing the host microorganism (Demain and Vaishnav, 2009). Characteristics of recombinant protein production are presented in Table 2.2 (Liu, 2013).

In this study, for the production of thermostable glucose isomerase, recombinant *E. coli* and *P. pastoris* host microorganisms carrying *xylA* gene inserted plasmids were used.

2.2.1 A Methytlotrophic Yeast: *Pichia pastoris*

Certain yeasts which have the ability to utilize methanol as a sole carbon and energy source were described by Koichi Ogata in 1969. Media and protocols for the cultivation of *Pichia pastoris* at high cell densities (>130 g/dry cell weight) on methanol was first developed by The Phillips Petroleum Company. *P. pastoris* having the ability to utilize methanol was considered as a single cell protein producer

in 1970s. However, in 1970s, due to oil crisis that caused significant increase in methane cost, production of single cell protein became inconvenient with *P. pastoris.* Afterwards, consensus of the company and Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA) was developing *P. pastoris* as a heterologous protein producer. Thereafter, researchers of SIBIA developed protocols, created vectors and plasmids for genetic engineering of *P. pastoris*. Following the isolation of alcohol oxidase (*AOX*) promoter and gene, genetic knowledge and fermentation procedures that had developed for single cell protein production were assembled. Then, in 1993 *P. pastoris* expression system was sold to its current patent holder Research Corporation Technologies (Tucson, AZ) and Invitrogen Corporation (Carlsbad, CA) was licensed to sell the system (Cereghino and Cregg, 2000).

Table 2.2 Characteristics of recombinant protein production by some host organims (Lui, 2013).

	Host Organism				
Characteristic	*E. coli*	*S. cerevisiae*	*P. pastoris*	*Insect*	*Mammalian*
High growth rate	Excellent	Very good	Very good	Poor-fair	Poor-fair
Availability of genetic systems	Excellent	Good	Fair	Fair-good	Fair-good
Expression levels	Excellent	Very good	Excellent	Good-excellent	Poor-good
Low-cost media available	Excellent	Excellent	Excellent	Poor	Poor
Protein folding	Fair	Fair-good	Fair-good	Very good-excellent	Excellent
Simple glycosylation	No	Yes	Yes	Yes	Yes
Complex glycosylation	No	No	No	Yes	Yes
Low levels of proteolytic degradation	Fair-good	Good	Good	Very good	Very good
Excretion or secretion	Poor normally, very good in special cases	Very-good	Very good	Very good	Excellent
Safety	Very good	Excellent	Very good	Excellent	Good

A methylotrophic yeast, *P. pastoris* can be genetically modified easily for the expression of recombinant proteins with both research and industrial purposes (Higgins and Cregg, 1998). In a diverse range of products from human endostatin to spider dragline silk, over 400 proteins have been produced by *P. pastoris* (Cereghino and Cregg, 2001).

2.2.1.1 Expression System of *P. pastoris*

P. pastoris expression systems are more expensive and usually time consuming when compared to *E. coli* or other equivalent expression systems. However, cost of recombinant protein production can be reduced right after the proper production system is established. Especially for the production of therapeutic proteins where the post-translational modifications are required, *P. pastoris* becomes an attractive alternative to insect or mammalian cells in terms of process economy (Vermasvuori *et al.*, 2009).

Advantages of *P. pastoris* expression system can be stated as: high cell density fermentation on minimal basal salt medium (Brierley *et al.*, 1990; Cregg and Higgins, 1995) secretion of less endogenous proteins, more recombinant product (Digan *et al.*, 1988; Laroche *et al.*, 1994), efficient post-translational modifications required for the activity of the protein (Digan *et al.*, 1988; Tschopp *et al.*, 1987), presence of strong well regulated promoter that is induced by methanol (Cregg and Madden, 1988; Cregg and Vedvick, 1993). Advantages and disadvantages of *P. pastoris* as an expression system are presented in Table 2.3.

Table 2.3 Advantages and disadvantages of *P. pastoris* as an expression system (Cregg *et al.*, 1999).

Advantages	Disadvanteges
Culturing Rapid growth rate High-cell density: > 100 g (DCW)/liter Clean medium composed of salts, biotin, and carbon source Uncomplicated scale up to large volume, high-density fermentor cultures	Fermentor culturing often needed to achieve high level of foreign protein
Molecular genetics Classical genetic methods available Molecular methods similar to *S. cerevisiae* Stable integrative expression vectors	Range of vectors limited
Promoters *AOXl*: strong, tightly regulated, and easily controlled; *GAP*: strong constitutive	
Expression Eukaryotic environment aids folding of higher eukaryotic foreign proteins; High expression levels	Improper post-translational modifications Native signals are not always processed Some proteins are misfolded and become stuck in secretory pathway Lower eukaryotic (high mannose)-type glycosylation
Secretion Proper post-translational modifications: Sulfhydril bond formation, signal sequence processing, folding, glycosylation High levels~g/liter fermentor cultures Few yeast proteins in medium--high initial purity of foreign protein	Proteases in medium degrade some foreign proteins

2.2.1.2 *AOX* and Other Promoters in *P. pastoris*

The first step in methanol utilization pathway of *P. pastoris* is catalyzed by the enzyme alcohol oxidase (*AOX*, EC 1.1.3.13) (Hartner and Glieder, 2006). *P. pastoris* has three phenotypes in terms of methanol utilization:

I.   Mut$^+$ (methanol utilization plus): contains both *AOX1* and *AOX2* genes that encodes two enzymes having *AOX* activity (Cereghino and Cregg, 2000). Total soluble proteins in extracts of the yeast can compromise *AOX1* up to

30% (Hartner, 2006; Couderc and Baratti, 1980; Macauley *et al.*, 2005) which is an indication of intensity of *AOX1* promoter (*pAOX1*). Unlike *AOX1*, *AOX2* promoter has a weaker activity. It has 15% of overall *AOX* activity of the cell (Cregg *et al.*, 1989).

II.   Mut^S (methanol utilization slow): contains only *AOX2* gene, *AOX1* is knocked out,

III.   Mut$^-$ (methanol utilization minus): both *AOX* genes are knocked out. Therefore, a Mut$^-$ cell cannot utilize and grow on methanol as the sole carbon source (Cereghino and Cregg, 2000).

Generally Mut$^+$ phenotypes have higher cell growth and protein production due to having a stronger promoter than Mut^S phenotypes (Romanos *et al.*, 1992;.van den Burg *et al.*, 2001; Kim *et al.*, 2009; Chiruvolu *et al.*, 1997). In the presence of some carbon sources like glucose and glycerol, methanol utilization pathway is repressed. When the medium is free of these carbon sources, protein production of both Mut$^+$ and Mut^S phenotypes can be induced by methanol (Cregg *et al.*, 1987). Advantages and disadvantages of *AOX* promoter are presented in Table 2.4 (Macauley-Patrick *et al.*, 2005).

Recently there have been some attempts to find an alternative promoter to prevent the usage of methanol during bioprocess. For this purpose, glyceraldehyde 3-phosphate dehydrogenase promoter (*PGAP*) and the formaldehyde dehydrogenase promoter (*PFLD1*) were developed as alternatives to *AOX* promoter (Cereghino and Cregg, 2000). *GAP* promoter is a constitutive promoter which is used for production of several heterologous proteins. It offers strong expression of recombinant proteins consitutively on glucose at comparable levels to *AOX1* (Waterham *et al.*, 1997). The enzyme encoded by *FLD1* gene has a role in methanol catabolism and methylated amines metabolism. Therefore, both methanol can be used as the sole carbon source and methylamine can be used as the sole nitrogen source for the induction of protein production (Cereghino, 2000). In addition, the promoter of isocitrate lyase (ICL1) gene can be considered as a good alternative since it is regulated by the carbon source within the culture (Menendez *et al.*, 2003).

Table 2.4 Advantages and disadvantages of *AOX* promoter (Macauley-Patrick *et al.*, 2005).

Advantages	Disadvantages
-Tight regulation and control of foreign protein expression by a repression/derepression mechanism.	-Since online probes are not sufficient and offline measuring is complicated, methanol monitoring during the process is an issue.
-Possibility of high level secretion of proteins that are toxic to the cells	-Storage of fire hazard methanol in large amounts is not favorable.
-Obtaining cells at high densities by starting with a repressing carbon source for only cell growth then switching it to methanol so transcription can be started.	-Since methanol is obtained from petrochemical sources, using it in food processes can be problematic.
-Expression of the protein can be induced by methanol easily	-Two carbon sources are required in order to switch one carbon source to another at an exact time

2.2.1.3 Methanol and Sorbitol Metabolism

When *Pichia* cells grow on methanol, some of the enzymes which have roles in methanol metabolism are found at abundant levels (Veenhuis *et al.,* 1983; Egli *et al.*, 1980). Veenhuis *et al.* (1980) reported that in order to utilize methanol, an atypical metabolic pathway was required together with particular enzymes. Alcohol oxidase (*AOX*) is the first enzyme in methanol catabolism of *P. pastoris*. It catalyzes the oxidation reaction of methanol to formaldehyde and hydrogen peroxide. Later, hydrogen peroxide is converted to oxygen and water with the catalytic activity of catalase (Figure 2.7). *AOX* is located in peroxisomes together with catalase. Some of

the formaldehyde is converted to carbon dioxide and formate by two cytoplasmic dehydrogenases, whereby the energy is obtained when growing on methanol.

Cellular components are generated by assimilation of remaining formaldehyde by a cyclic pathway. This cyclic pathway begins with the condensation reaction occurring between formaldehyde and xylulose 5-monophosphate catalayzed by dihydroxyacetone synthase (DHAS) which is also an enzyme located in peroxisomes. Glyceraldehyde 3-phosphate and dihydroxyacetone are produced in consequence of the reaction and leave the peroxisomes. Xylulose 5-monophosphate is generated upon entry of glyceraldehyde 3-phosphate and dihydroxyacetone into a cytoplasmic pathway. Glyceraldehyde 3-phosphate is generated by every three cycles. *AOX* and DHAS can be found abundantly in the cells that utilize methanol; cannot be found where other carbon sources such as glucose, glycerol or ethanol are utilized. Induced levels of *AOX* enzyme can be found in the cells that are fed with methanol at growth limiting conditions (Couderc and Baratti, 1980; Roggenkamp *et al.*, 1985).
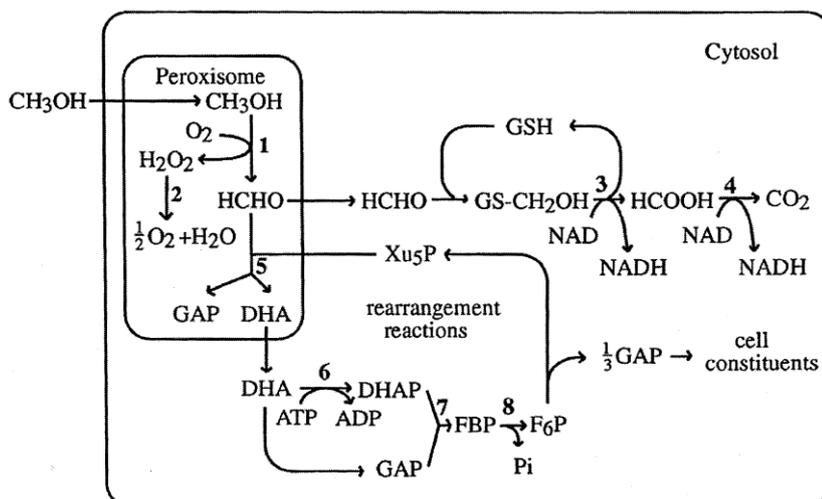
Figure 2.7 Methanol utilization pathway of *P. pastoris* (Cereghino and Cregg, 2000). 1. *AOX*; 2. Catalase; 3. formaldehyde dehydrogenase; 4. formate dehydrogenase, 5. dihydroxyacetone synthase; 6. dihydroxyacetone kinase; 7. fructose 1,6-biphosphatealdolase; 8. fructose 1,6-bisphosphatase.

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As stated before, *P. pastoris* can consume methanol as a carbon source but cannot tolerate high concentrations of methanol since generation of toxic components, formaldehyde and hydrogen peroxide, is seen inside the cell during methanol utilization pathway (Couderc and Baratti, 1980; Cregg and Madden, 1988; Van der Klei *et al.*, 1990). Therefore, in order to maintain methanol concentration at low levels, semi (fed)-batch feeding strategy is used, generally (Zhang *et al.*, 2000).

To increase the recombinant protein production by *P. pastoris*, multiple carbon sources have been examined so far. (Cregg *et al.*, 1993; Loewen *et al.*, 1997; Katakura *et al.*, 1998; Zhang *et al.*, 2003). Sorbitol is a considerable carbon source that is co-fed with methanol owing to its non-repressing characteristics on *AOX1* promoter (Helene *et al.*, 2001;.Xie *et al.*, 2005).

In sorbitol utilization pathway of yeasts (Figure 2.8), sorbitol is converted to D-fructose by D-glucitol dehydrogenase and enters mannitol cycle. Afterwards, following the conversion to fructose-6-phosphate by fructokinase, it enters glycolysis pathway (Walker, 1998).

2.2.1.4 Post-Translational Modifications and Protein Secretion

Typical post-translational modifications such as signal sequence processing, folding, disulphide bridge formation, lipid addition, O-and N-linked glycosylation performed by high eukaryotes can be achieved by *P. pastoris*. Proteins can be expressed both intracellularly and extracellularly by *P. pastoris*. Since yeast secretes little amounts of endogenous proteins, extracellular production of recombinant proteins can be achieved by the activity of signal sequences. Nevertheless, in terms of stability and proper folding of recombinant protein, secretion is preferred for foreign proteins that are already secreted by their native host microrganism (Cereghino and Cregg, 2000).
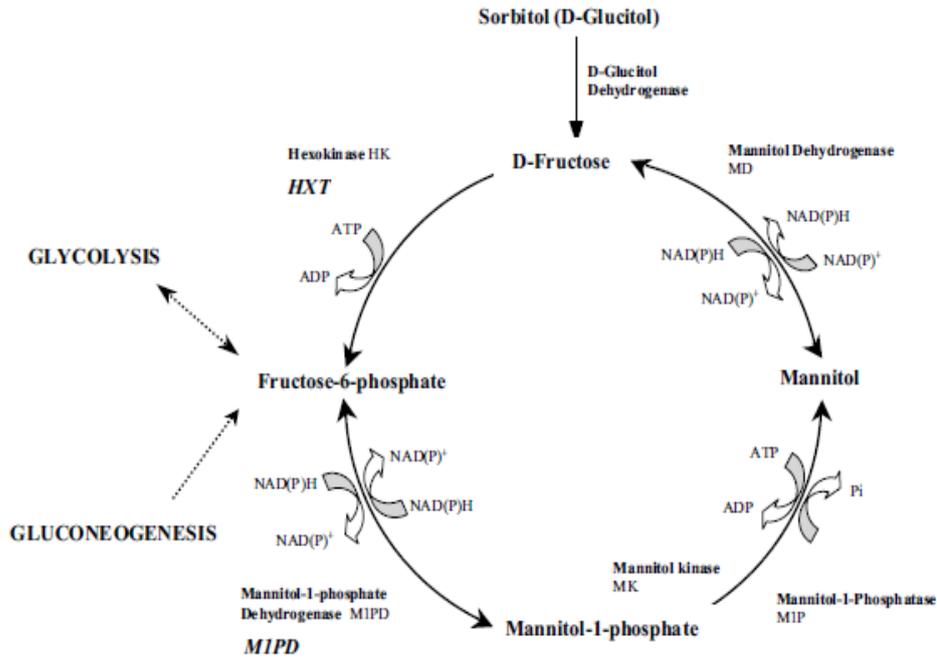
Figure 2.8 Sorbitol and mannitol utilization in yeasts. 1, D-glucitol dehydrogenase; 2, D-fructokinase; 3, mannitol-phosphate dehydrogenase; 4, mannitol-1-phosphatase; 5, mannitol dehydrogenase (Walker, 1998).

2.2.1.5 Proteolytic Degradation

Proteolytic degradation is one of the most important issues in recombinant protein production by *P. pastoris* (Idiris *et al.*, 2010). While some products expressed by *P. pastoris* keep their functionality, some proteins lose their function depending on their sensitivity to proteolytic degradation. Sensivity to proteases mostly depends on amino acid sequence. Futhermore, three dimensional structure may hide or reveal recognizable cleavage sites. Some proteases that can be intracellular, extracellular or bound the cell such as serine-type, aspartic acid and cysteine are seen during the cultivation of *P. pastoris* (Potvin *et al.*, 2012).

2.2.2 A Workhorse for Recombinant Protein Production: *Escherichia coli*

Escherichia coli is an enteric Gram-negative, rod-shaped bacterium, containing a circular genome at the size of 4.6 Mb (Blattner *et al.*, 1997). Simplicity of genetic-manipulation of *E. coli* speficied its role as host microorganism for manipulation and characterization of recombinant DNA. *E. coli* has been subjected to researchs intensively during the past 60 years (Casali, 2003). Due to the ability of growing to high cell densities on low-priced media and well studied genetic characteristics, *E. coli* is frequently used for recombinant protein production (Paliy *et al.,* 2007). As an expression system, *E. coli* has the advantage of easy growing conditions, rapid biomass formation and uncomplicated scale-up procedure (Baneyx, 2004). *E. coli* expression system is mostly preferred for the expression of small, un-modified and soluble proteins and more than half of the products of pharmaceutical companies are produced by *E. coli* (Jayaraj and Smooker, 2009).

Using growth media such as Luria broth (LB) (Curless *et al*., 1990; Li *et al*., 1999), synthetic M9 minimal medium (Lim *et al*., 2000), terrific broth (TB) (Zanette *et al*., 1998; Lim *et al*., 2000) and super broth (SB) (Madurawe *et al*., 2000) high cell densities can be reached during *E. coli* fermentation.

2.2.2.1 Characteristics of *E. coli* BL21

The strain BL21 is a derivative of *E. coli* B strain which is particularly constructed with the aim of high level expression together with key genetic markers and an induction system. *E. coli* BL21 (DE3) pLysS strain which is a derivative of *E.coli* BL21 was used in this study. *E. coli* BL21 (DE3) pLysS strain contains an expression system with DE3 bacteriophage λ lysogen carrying *lacI* gene under the control of *lacUV5* promoter for expression of T7 RNA polymerase. Basal expression level of the related gene is inhibited with pLysS plasmid by encoding T7 lysozyme (http://tools.invitrogen.com).

2.2.2.2 *Lac* Operon and T7 Expression System

Even though for the protein expression in *E. coli*, numerous promoters are described, a few of them are used. Properties of a practical promoter are strength, having low of level basal expression or tight regulation, ease of transfer to other strains of *E. coli*, offering a simple, cost-effective expression, independence from mostly used ingredients of culture media (Terpe, 2006). Intracellular recombinant proteins accumulation inside the cells can be achieved up to 40-50% of total protein (Makrides, 1996).

Usually gene overexpression is regulated by strong promoter systems such as *lac* promoter and its derivatives *lacUV5*, *tac* and *trc* promoters (Amann *et al*., 1988; DeBoer *et al*., 1983). The natural inducer of *lac* or *lac* derived promoters is allolactose which is formed by *E. coli* cell via transglycosylation of lactose. Presence of glucose can cause catabolic repression (Ishizuka *et al*., 1993; Neubauer *et al*., 1991) subsequently hindering lactose induction. In practical induction of *lac* or *lac* derived promoters, a non-hydrolyzable analog of lactose, isopropyl-β-D-thiogalactopyranoside (IPTG) is preferred. Unlike lactose, IPTG is not metabolized as a carbon source by the cell. Therefore, it offers a consistency in terms of induction (Narayanan *et al.*, 2006).

However, IPTG induction system has some obstacles compared to lactose induction (Jonnason *et al*., 2002). Its cost and potential toxicity which can be problematic in downstream operations of large-scale production are major drawbacks of this system. Furthermore, inclusion body formation occurs usually using IPTG-induction (Lin *et al*., 2001). Another alternative inducer of *lac* or *lac* derived promoters is galactose (Barkley *et al*., 1975). In literature, it was stated that to induce *lac* promoter for recombinant protein production, it was not a requierement to add IPTG when beat molasses used as carbon source since it contained galactose, which was one of the monomers of trisaccharide raffinose, in it. Recombinant protein production levels was the same with or without IPTG addition. Therefore, it was concluded that induction efficiency of galactose which was known as strain

dependent, was sufficient for induction of *lac* promoter of *E. coli* BL21 (DE3) pLysS (Çalık and Levent, 2009).

The *lac* operon is used for lactose utilization and contains three genes which are *lacZ*, *lacY* and *lacA*. In the upstream a promoter, operator and *lacI* gene are located. *LacI* gene is transcribed separately from other genes. Operator is where *LacI* transcriptional repressor is bound. The first gene encodes for β-galactosidase which produces glucose and galactose from lactose. Then, the second gene encodes the enzyme, lactose permease which is used for the transfer of the β-galactosidase into the cell (Zabin, and Fowler, 1980). The product of the last gene is thiogalactoside transacetylase (Lewendon *et al.*, 1995). The expression of these proteins is required as the bacterium utilizes lactose as a carbon source and regulated with respect to lactose and glucose concentrations. When there is no lactose in the medium, *lacI* repressor binds to operator and inhibits binding of RNA to the promoter. When there is lactose in the medium, it loses its affinity towards operator (Miller, 1980). The *lac* operon is also regulated with glucose by catabolite gene activator protein (CAP) which is encoded by *crp*. It binds cAMP and *lac* promoter to enhance transcription by RNA polymerase. Absence of glucose in the medium leads to cAMP production, therefore binding of CAP to promoter and expression (Emmer *et al.*, 1970; Zumar *et al.*, 1970). The structure and regulation of *lac* promoter is presented in Figure 2.9.

T7 expression system is frequently used for the production of desired proteins in *E. coli*. T7 RNA polymerase gene in the chromosome of *E. coli* BL21 (DE3) is under the control of *lacUV5* promoter. Since T7 polymerase is so selective and active, it can be used for the production of diverse recombinant proteins (Studier, 1986). In T7 expression system, gene that is aimed to be expressed is cloned at the downstream of the T7 promoter. Since target gene is independent from host's own RNA polymerase, basal expression of the gene does not occur when there is no T7 RNA polymerase (Durbin, 1999). Production of recombinant granulocyte colony-stimulating factor (G-CSF) by *E. coli* using T7 expression system is shown in Figure 2.10.
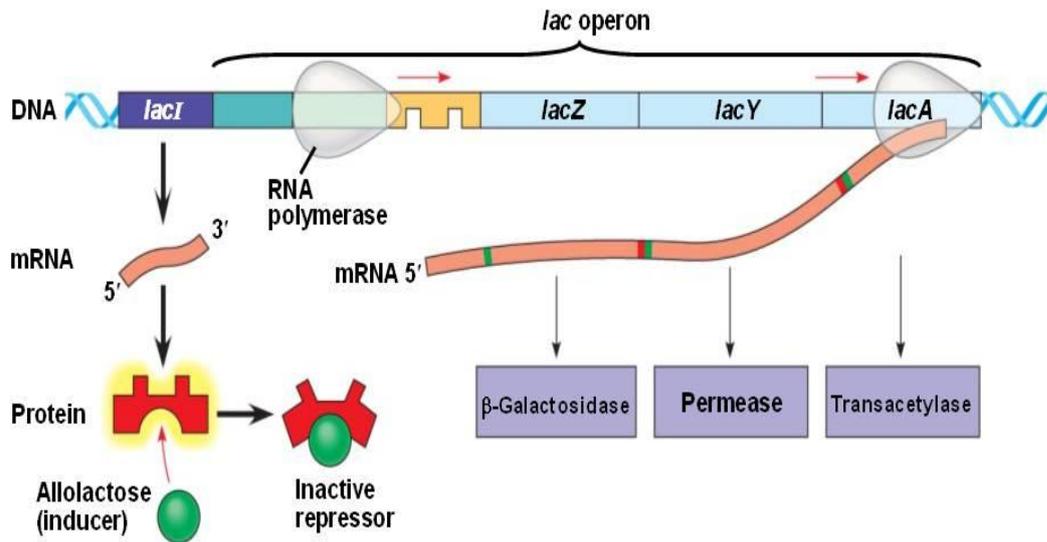
Figure 2.9 Structure and regulation of *lac* promoter (Retrieved from
http://bio1151.nicerweb.com/Locked/media/ch18/lac_induced.html).
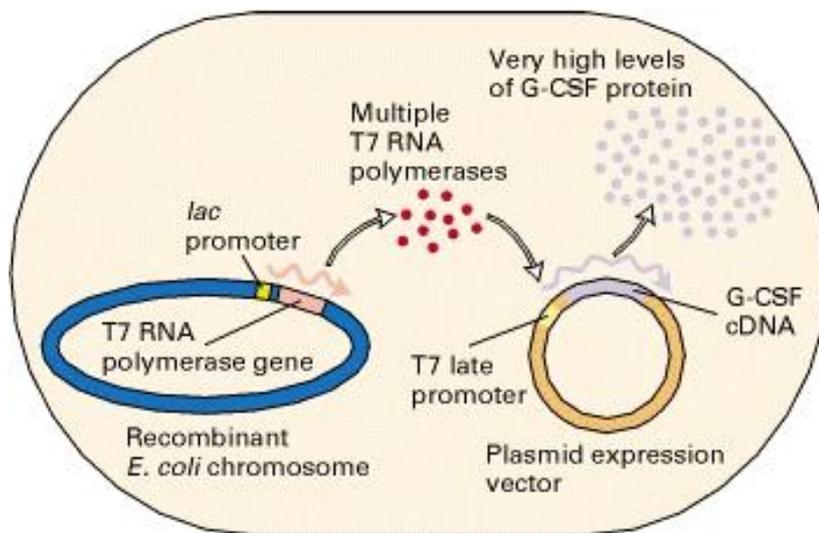
Figure 2.10 Production of granulocyte colony-stimulating factor production using
T7 expression system (Retrieved from http://www.biotechspace.site90.com/?p=227).

2.3 Genetic Engineering Techniques and Methodology

The milestone of molecular biology technologies addresses the gene. Isolation and amplification of a gene can be achieved by cloning the gene via inserting into a distinct DNA molecule which acts as an agent of replication in living cells of organisms. If two DNA molecules obtained from distinct sources are combined, the resulting DNA molecule is refered as recombinant DNA. Then, the recombinant DNA is inserted into a host microorganism which ensures the amplification of foreign DNA by producing its clone. Afterwards, it is purified for further analysis (Mullis, 1990).

With the purpose of obtaining recombinant DNA products, first; the gene of interest is replicated using polymerase chain reaction (PCR) together with specific primers containing restriction enzyme recognition sites. Afterwards, gene and the vector are cut by the same restriction enzyme. Since they are cut with the same enzyme, they recongnize each other from those sites. Using the enzyme ligase, they stick to each other. After ligation, the cloning vector, usually plasmids, that carry antibiotic resistance genes, are transferred to the host microorganism. Due to the antibiotic resistance genes present in the plasmid, recombinant cells are able to grow on antibiotic containing medium. Afterwards, colonies grown on medium are selected for further analysis.

Polymerase Chain Reaction (PCR)

Bacterial cells that contain a cloned gene can be used to amplify it in number. However, numerous DNA fragments at first are required at the beginning. Polymerase chain reaction (PCR) which was discovered by Kary Mullis in 1983 gave the opportunity by replicating DNA samples in a test tube containing mixture of DNA, four nucleotides (A,G,C,T), DNA polymerase and specific primers (Liu, 2013).

The main steps of PCR reaction are denaturation, annealing and extention. The process begins with denaturation of DNA at high temperatures. It continues with the annealing of two primers to the DNA template strands which is required for extention. In last step, extention starts at the end of annealed primers with the help of an enzyme Taq polymerase to produce complementary copy of DNA. It synthesizes two new DNA strands by using the original DNA as a template. The process is repeated and original DNA is duplicated in each cycle (Ochman *et al.*, 1988). Main steps of PCR are shown in Figure 2.11 (Liu, 2013).
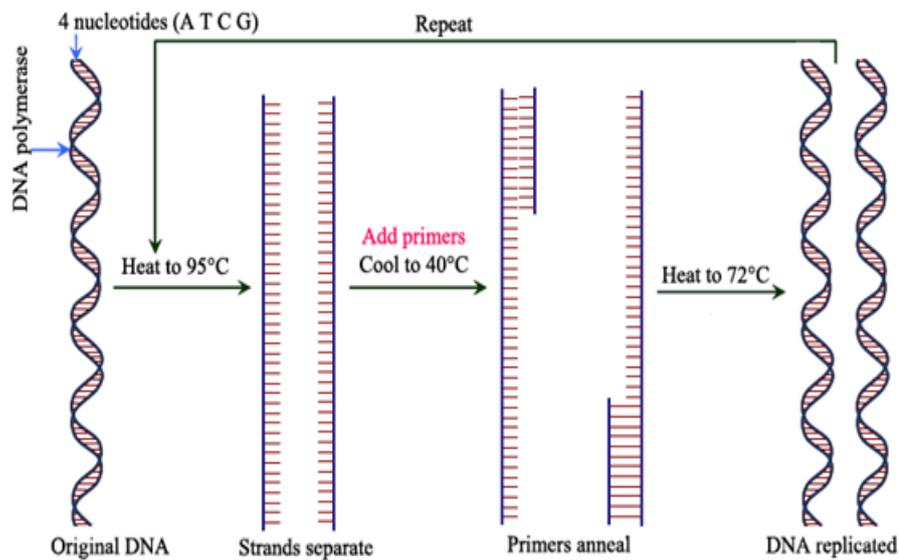
Figure 2.11 Steps of polymerase chain reaction (Liu, 2013).

Restriction Digestion and Ligation

Arber (1965) reported the restriction modification system of bacteria which was used with the purpose of defense against a threat, bacteriophage. A restriction enzyme specifically binds onto the DNA molecule from a recognition sequence, leads the subsequent cleavage at phosphate backbone of the DNA molecule. With the advent of these enzymes, researchers in biotechnology field are able to cut DNA

molecules into fragments with the purpose of analyzing and constructing new molecules of DNA (Yoshikawa and Isono, 1991).

One fragment of double stranded DNA can be transferred to another following restriction enzyme digestion and ligation in a simple way. After the cleavage of DNA, DNA product is obtained as either 5' or 3' overhangs (sticky ended) or blunt ended (Invitrogen). A schematic representation of restriction digestion is presented in Figure 2.12.

DNA ligase is used to repair cut DNA and by joining two complementary fragments which is the reverse of restriction digestion. Therefore, DNA fragments obtained from different sources can join together (Figure 2.13) (Liu, 2013).
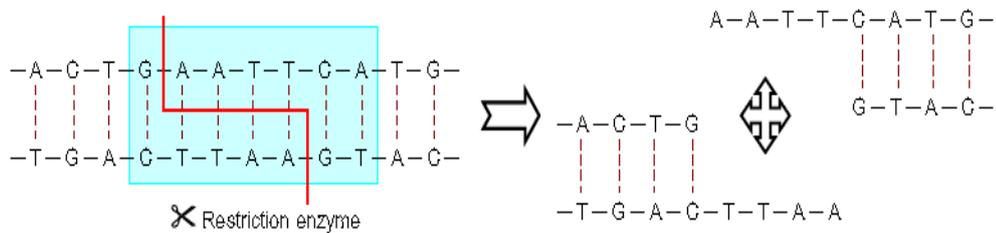
Figure 2.12 Restriction enzyme digestion of DNA (Liu, 2013).
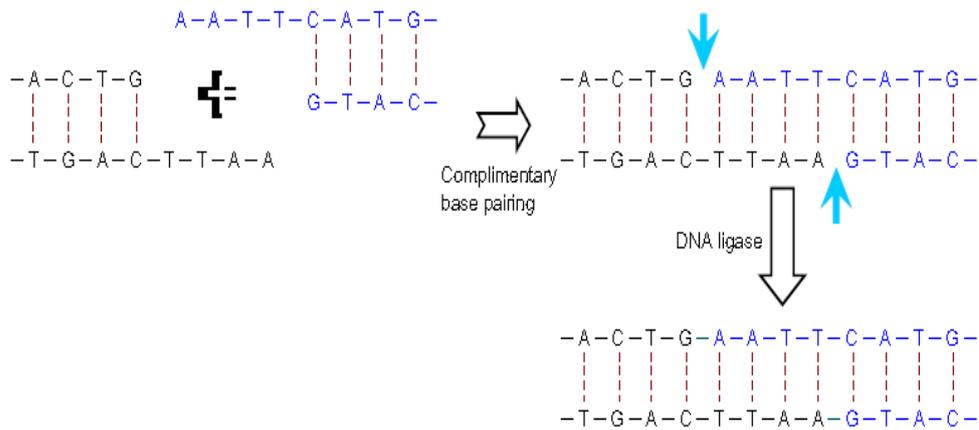
Figure 2.13 Ligation after restriction enzyme digestion (Liu, 2013).

Plasmids

Plasmids usually have three regions in common: (i) Origin of replication (ii). Selective marker (i.e. antibiotic/drug resistance gene). (ii) Multiple cloning site for insertion of foreign gene (Lodish *et al.,* 2003).

pPICZα-A vector (Figure 2.14) contains *AOX1* promoter region for regulation and induction of recombinant protein expression, thirteen unique restriction sites where the gene is inserted, α-factor signal sequence for protein secretion, Zeocin resistance gene, a polyhistidine (6xHis) peptide, Factor Xa to make purification of recombinant protein easier, TEF1, EM7 promoters, pUC origin for replication and maintenance of plasmid and CYC1 transcription termination region. (Invitrogen, Life Technologies,Cat. No: V195-20).

pPICZ-A containing intracellular *xylA* gene was constucted by Ata (2012). First, α-factor signal sequence was removed. Then, digestion of plasmid with a restriction enzyme and ligation of *xylA* gene which was digested with the same restriction enzyme was performed. The constructed plasmid pPICZ-A::*xylA*$_{int}$ was used for *Pichia* transformation. Since it does not contain α-factor signal sequence, the *xylA* gene is expressed intracellularly using pPICZ-A::*xylA*$_{int}$.

pRSETA vector (Figure 2.15) contains a strong bacteriophage T7 promoter which regulates the expression of the cloned gene. Expression of the gene is induced by T7 RNA polymerase produced in *E. coli* BL21 (DE3). It consists of ampicillin antibiotic resistance gene, N-terminal polyhistidine (6xHis) tag for easy purification of recombinant protein and enterokinase cleavage site for removal of the fusion tag, multiple cloning site with eleven restriction enzyme recognition sequences, f1 and pUC regions (Invitrogen, Life Technologies, Cat. No: V351-20).

pRSETA containing *xylA* gene was constucted by Angardi (2013) via digestion of the plasmid and gene of interest with the same restriction enzyme and subsequent ligation. In feeding strategy development experiments for thermostable GI production, *E. coli* carrying pRSETA::*xylA*$_{int}$ was used.
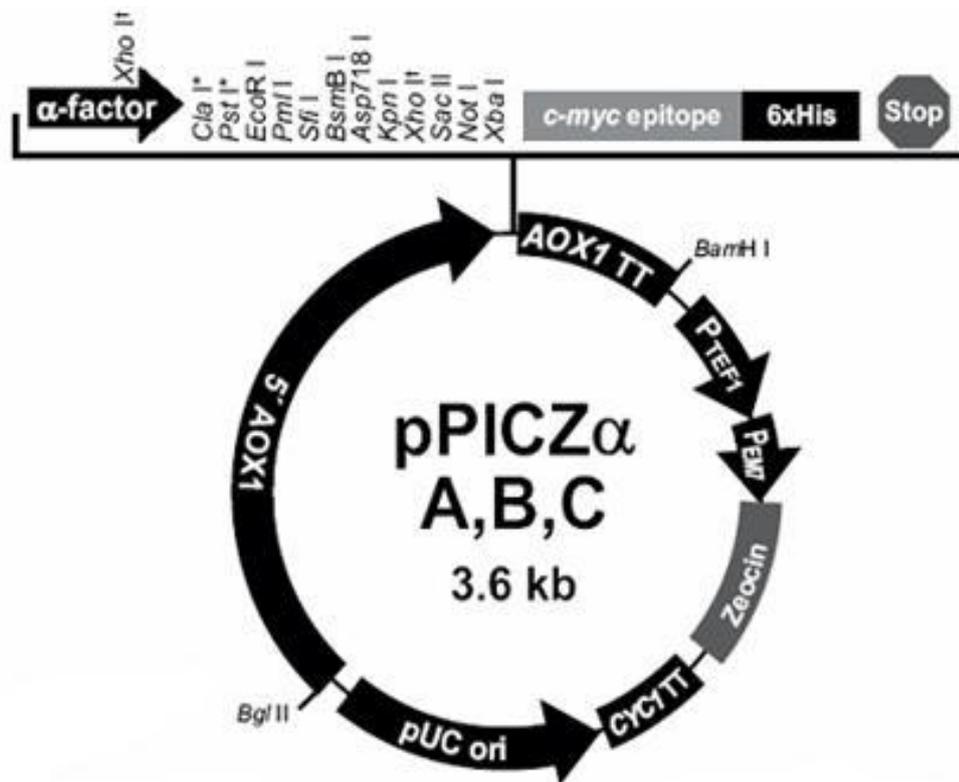
Figure 2.14 pPICZα-A cloning vector (Invitrogen, Life Technologies,Cat. No: V195-20).
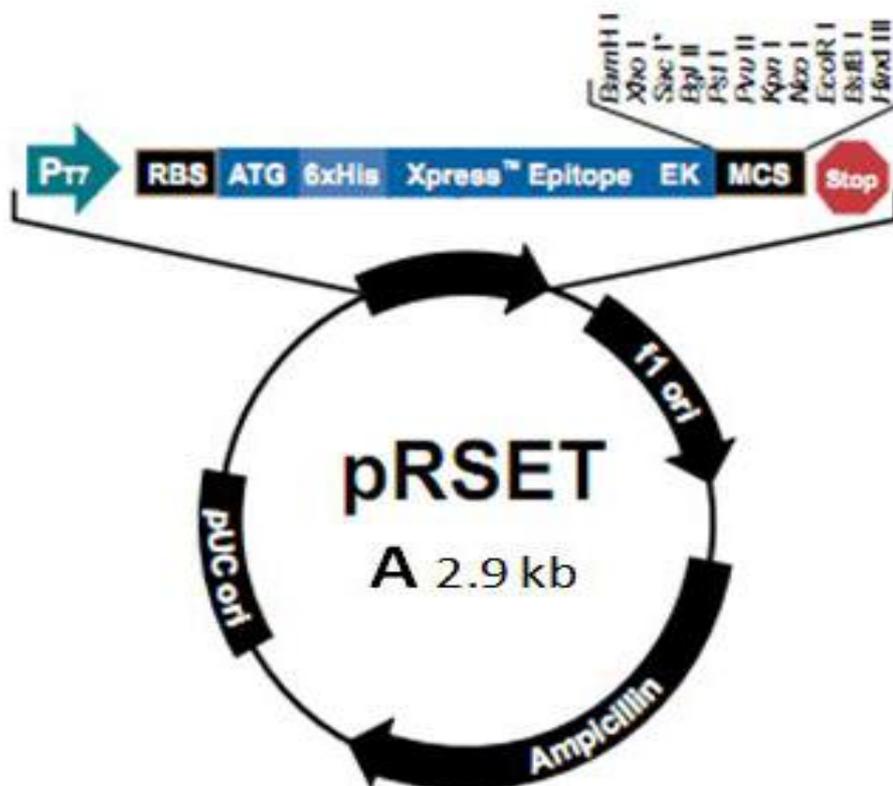
Figure 2.15 pRSETA cloning vector (Invitrogen, Life Technologies, Cat. No: V351-20).

Earlier, transformation of yeast cells were carried out using spheroplasts which were stabilized in an isotonic environment. Even though spheroplasts are used broadly, it is a time-consuming method together with the disadvantage of dependent efficiency on cell regeneration of protoplasts (Heslot and Gaillardin, 1991). In 1983 Ito *et al.* transformed intact yeast cells with lithium salt treatment for the first time. In the protocol, cells were subjected to lithium chloride, after the addition of plasmid DNA which contained gene of interest and polyethylene glycol (PEG) heat shock was applied. 400 transformants per µg DNA was achieved with this method. The protocol is less efficient from other current protocols for yeast spheroplast transformation (Broach *et al.*, 1979; Struhl *et al.*, 1979). However, advantageous side of this protocol is being more straightforward and not requiring cell incubation on regeneration agar. Among the works aiming the increase in efficiency of this

protocol, using sonicated carrier DNA reached the efficiency of 10000-20000 per µg DNA (Gietz and Sugino, 1988; Steams *et al.*, 1990). In 1991, Baker stated that using carrier DNA resulted the increased recovery of transformants from agar. Transformation with electroporation was used together with spheroplasts (Karube *et al.*, 1985) and the entire cell (Hashimoto *et al.*, 1985). Since the usage of PEG is eliminated in this method, cell damage caused by PEG can be avoided.

Integration of DNA molecule into the genome of yeast occurs by homologous recombination in the transformation process. The efficiency of transformation was investigated using both linearized plasmid DNA and circular plasmid DNA. When the integrating plasmid DNA was cut by a restriction enzyme where it had homology to yeast genome, the efficiency of transformation was increased 10-1000 fold since the two ends of the linearized plasmid worked together while single reciprocal crossover occured with circular plasmid in integration process (Orr-Weaver *et al.*, 1981). In addition, it was reported that cloning of *Pichia pastoris* via homologous recombination between linearized plasmid DNA containing inserted gene and genome of *P. pastoris* created stable transformants (Cregg *et al.*, 1985; Cregg *et al.,*1989).

2.4 Bioreactor Operation Parameters

To a certain extent, products can be developed in laboratory. However, in order to adapt modern biotechnology to industry, it requires engineering skills and know-how. Although the biological systems are complex and hard to control, they obey the laws of physics and chemistry which make them suitable for engineering analysis. Therefore, bioreactor design and operation, sterilisers, downstream and recovery equipment, automation and control, safety of fermentation are important things to be considered in engineering aspects. In bioprocess development, after the genetic modification of host microorganism, growth and production properties of cells are investigated in shake flasks. After determining pH, temperature and the composition of the medium which gives the optimal growth and productivity, parameters such as specific cell growth rate, specific productivity and yield are calculated to describe

performance of microorganisms. Thereafter, it is proceeded to the second step: bioreactor operation. Due to consisting of the equipments for measuring and adjusting temperature, pH and dissolved-oxygen, bioreactors offer better control of process variables than shake flasks (Doran, 2012).

2.4.1 Medium Design and Composition

The growth medium composition has a considerable metabolic effect on both cell and protein production. For instance, mRNA translation is related with the temperature and medium composition (Corisdeo and Baiyang, 2004). Complex media generally offer higher cell growth and protein productivity than chemically defined media (Zanette *et al.*, 1998). Some carbon and nitrogen sources used in industrial fermentations are presented in Table 2.5 (Dunn, 1985). Chemically defined media are usally preferred for recombinant protein production (Lim and Jung, 1998; Cserjan-Puschmann *et al.*, 1999; Zhang and Greasham, 1999; Kweon *et al.*, 2001) as they offer consistency in the production process and easiness in downstream processing of related protein (Tripathi *et al.,* 2009).

For high cell density *Pichia pastoris* fermentations, basal salt medium (BSM) is used, frequently (Pichia fermentation process guidelines, Invitrogen). Supplementation of micronutrients to fermentation medium was done by adding trace salt solution PTM1 (Invitrogen, 2002). In *Pichia* fermentations, the first aim is to increase cell concentration using the carbon sources like glycerol or glucose which repress *AOX* promoter. Since the recombinant protein production is growth related, optimum biomass concentration, that is to be reached before the beginning of induction, should be investigated in order to produce large amounts of recombinant proteins (Macauley-Patrick, 2005). Nitrogen sources like yeast extract and casamino acids increased secretion and accumulation of recombinant proteins. (Sreekrishna *et al.*, 1997). In addition, EDTA and L-arginine enhanced the accumulation of single-chain antibody (Shi *et al.*, 2003). The most common way of fullfiling nitrogen demand and overcoming nitrogen limited conditions is using ammonium ions in the fermentation of *P. pastoris*. Since it was reported that low ammonium concentrations

within the fermentation medium could cause degradation while higher concentrations inhibited the production of recombinant hirudin (Yang *et al.,* 2004), concentration of the nitrogen source should be considered carefully. It was reported that carbon source could have an impact on recombinant protein productivity both for proteins retained inside the cell and secreted (Hohenblum *et al.*, 2004). After the investigation of different carbon sources in *Pichia* fermentation by Inan and Meagher (2001), it was reported that sorbitol, mannitol, alanine or trehalose did not have repressing effects on *AOX1* promoter and could be used as carbon source together with methanol as inducer.

For high cell density *E. coli* fermentations, such mediums as Luria broth (LB) (Curless *et al*., 1990; Li *et al*., 1999), synthetic M9 minimal medium (Lim *et al*., 2000), terrific broth (TB) (Zanette *et al.,* 1998; Lim *et al*., 2000) and super broth (SB) (Madurawe *et al*., 2000) are used generally.

Table 2.5 Some of carbon and nitrogen sources used in industrial fermentations (Dunn, 1985).

Carbon source	Nitrogen source
Starch waste (maize and potato)	Soya meal
Molasses (cane and beet)	Yeast extract
Whey	Distillers solubles
n-Alkanes	Cottonseed extract
Gas oil	Dried blood
Sulfite waste liquor	Corn steep liquor
Domestic sewage	Fish solubles and meal
Cellulose waste	Groundnut meal
Carbon bean	

For *E. coli* fermentation, accumulation of nutrients has a negative effect on cell growth and recombinant protein production (Shiloach and Fass, 2005). High glucose concentrations in the fermentation medium causes the Crabtree effect and acetate accumulation that inhibits growth of *E. coli* (Panda, 2003). For high cell density fermentation of *E. coli*, most used carbon source is glucose. Salts such as phosphate, sodium, potassium, magnesium, ammonia and sulfates, trace elements and complex nitrogeneous compounds are used together with glucose usually. Since high nutrient concentrations can be problematic, as stated before, *E. coli* fermentations are carried out at starting with low concentrations of substrates followed by addition of nutrients in the growth process with suitable feeding strategy for reduced acetate formation (Babaeipour *et al.*, 2008). If the components are fed to the system regarding to consumption rate of the cell, nutrient accumulation and its adverse effects can be avoided.

When *E. coli* cells grow on glucose at aerobic conditions, 10-30% of the carbon flux is excreted to acetate. Acetate formation is more common in defined media than in complex media. Acetate production occurs in anaerobic fermentation conditions though, excess amounts of glucose can cause acetate formation in aerobic conditions. When the acetate concentration reaches higher values such as >5 g L^{-1} at pH 7, it decreases cell and product yield (Eiteman and Altman, 2006; De Mey *et al.,* 2007). Specific growth rate and composition of the medium affect acetic acid formation. When the specific growth rate exceeds 0.2 h^{-1} for complex media and 0.35 h^{-1} for defined media, acetate formation occurs (Lee, 1996; Panda, 2003; Luli and Strohl, 1990).

For the production of BAL by *E. coli* BL21 (DE3) pLysS, Kaya *et al.* (2009) developed a glucose-based medium that contains 8 g L^{-1} glucose, 5 g L^{-1} $(NH_4)_2HPO_4$ and salt solution and investigated the recombinant protein production and cell growth with respect to oxygen transfer conditions. Angardi and Çalık (2013) investigated the glucose isomerase production capacity of *E. coli* BL21 (DE3) pLysS using medium that contained and 5 g L^{-1} $(NH_4)_2HPO_4$ and salt solution either with 8 g L^{-1} glucose or 30 g L^{-1} molasses-based medium. Molasses-based medium followed by continuous molasses feeding was found to be superior to glucose-based medium and

continuous glucose feeding. Pre-determined feeding with specific growth rate of $\mu_0=0.05$ h^{-1} was reported as the best strategy among other pre-determined feeding values when it was used with 30 g L^{-1} molasses-based inital medium. Therefore, starting point of the current study was 30 g L^{-1} molasses-based medium which was followed by pre-determined feeding based on the specific growth rate of $\mu_0=0.05$ h^{-1}.

2.4.1.1 Molasses

Molasses is initially used to describe liquid obtained during the sucrose production process which contains repeated evaporation, crystallization and centrifugation of sugar cane and sugar beet juices. Beet molasses is a by-product of sucrose production from sugar beet (Curtin, 1983). The components of beet molasses are presented in Table 2.6 (Olbrich, 1963). Ingredients of the sugar beet molasses obtained from Ankara Sugar Factory for this study is presented in Table 2.7.

Molasses is a cheap raw material that can be found in large quantities. Since it has organic non-sugars, vitamins and essential minerals, it supports microbial growth and it is suitable to be used for ethanol production in sugarcane factories where it is obtained (Park and Baratti, 1991; Kirk and Othmer, 1994). Furthermore, with industrial purposes, molasses can be used for the production of acetic acid, vinegar, butanol/acetone, lactic acid, citric acid, glycerin as a raw material (Paturau, 1989).

Several studies have been performed for the production of diverse products by different microorganism using molasses as carbon source. Park and Baratti (1991) investigated the effect of molasses-based medium containing magnesium sulfate on ethanol fermentation carried out using *Zymomonas mobilis*. It was reported that cell growth and product yield was sufficient and cost of using yeast extract could be eliminated using molasses for fermentation.

In 1992, Diwany *et al.* used mud-free H_2SO_4 treated beet molasses for the production of ethanol by *Saccharomyces cerevisiae* Y-7 and showed that using

repeated fermentation technique with immobilized cells, molasses was superior to sucrose.

In 1994, Ghozlan investigated the riboflavin production ability of *Mycobacteriurn phlei* when beet molasses was used as the sole carbon source. High amount of riboflavin was obtained with the medium containing 9% of beet molasses, which was resin- treated with the aim of removing heavy metals and muddy residues.

Agarwal *et al.* (2006) reported utilization of cane molasses as a carbon source for succinic acid production by an isolate which was chosen after screening and identified as *E. coli*. Succinic acid production was developed and optimized using cane molasses and corn steep liquor in 500 ml sealed bottles. Optimization of fermentation medium yielded 9-fold increase in succinic acid production compared to the initial medium containing glucose and peptone. Later in 2007, Agarwal *et al.* optimized the fermentation medium for succinic acid production using response surface methodology. With this approach, the yield of succinic acid production (15.2 g L^{-1} in 36h) was doubled when compared to one-variable-at-a-time-approach (7.1 g L^{-1} in 36h) in 500 ml sealed bottles containing cane molasses under anaerobic conditions. Then, further developments resulted 26.2 g L^{-1} succinic acid production by *E. coli* in 30 h under optimized conditions.

Çalık and Levent (2009) investigated benzaldehyde lyase (BAL) production using pretreated beet molasses by recombinant *Escherichia coli* BL21 (DE3) pLysS. Different pretreated molasses concentrations were examined and the highest recombinant enzyme activity was obtained with C_{M0}=30 gL^{-1} of molasses in the fermentation medium which was 1.5 fold higher than the amount produced by Kaya-Çeliker *et al.* in 2009 on a glucose-based medium.

Ye *et al.* 2010 used untreated cane molasses in co-production of a carbonyl reductase (PsCR) and a glucose dehydrogenase (BmGDH) by *E. coli* Rosetta (DE3) pLysS strain. Since the researchers obtained a good cell growth on untreated cane molasses, the possibility of a sucrose hydrolase gene, which led to utilization of major carbon source of molasses, in the genome of this strain was suspected.

However, no genome-based experiment was carried out to prove the existence of the sucrose hydrolase gene.

In 2013, Angardi and Çalık used C_{M0}=30 g L^{-1} of pretreated molasses-based medium for the production of thermostable glucose isomerase. 16399 U L^{-1} enzyme activity was reported with molasses feeding in semi-batch operation based on a specific growth rate of 0.05 h^{-1}. Afterwards, In the M.Sc. study of Akdağ (2012), 35264.5 U L^{-1} enzyme activity was reported using untreated molasses for the production of thermostable glucose isomerase since the strain *E. coli* W which had the ability to utilize sucrose as a carbon source.

Table 2.6 Components of molasses obtained from European sugar beet samples (Olbrich, 1963)

Component	Average composition (%)
Water	16.5
Sucrose	51.0
Glucose and Fructose	1.00
Raffinose	1.00
Organic non-sugars	19.0
Ash components:	11.5
SiO_2	0.10
K_2O	3.90
CaO	0.26
MgO	0.16
P_2O_5	0.06
Na_2O	1.30
Fe_2O_3	0.02
Al_2O_3	0.07
CO_3	3.50
Sulfates as SO_3	0.55
Cl	1.60

Vitamins (mg/100 g)	
Thiamine (B1)	1.30
Riboflavin (B2)	0.40
Nicotinic acid	51.0
Ca-pantothenate (B3)	1.30
Folic acid	2.10
Pyridoxine-HCl (B2)	5.40
Biotin	0.05

Table 2.7 Composition of sugar beet molasses obtained from Ankara Sugar Factory

Dry solids (%)	83.04
Sucrose	51.07
Raffinose	0.67
Invert sugar	0.40
Total nitrogen	2.05
Organic non-sugars	16.16
Ash	12.69
Water (%)	16.69

2.4.2 Temperature

Growth and temperature relationship is dependent on strain. The temperature of the bioprocess should be maintained at the optimum value which is determined for the particular microorganism (Sonnleitner, 2000).

Wild type *E. coli* has an optimal temperature of 37°C (Holt *et al.*, 1994). *Pichia* fermentations are carried out at 30°C in an optimal manner (Wegner, 1983). Higher temperatures cause cell death and lysis and secretion of proteases into the fermentation medium (Inan *et al.*, 1999). In parallel, it is reported that low temperatures increase product yield providing proper folding of recombinant proteins (Macauley-Patrick *et al.*, 2005) and reduce cell lysis (Hong *et al.*, 2002). In this study, GI production was performed at 30°C for *P. pastoris* and 37°C for *E. coli*.

2.4.3 pH

pH is one of the variables that should be controlled in bioprocesses since cell metabolism is directly affected by it. pH of the medium is determined by potentiometric electrodes containing liquid or gel electrolytes (Sonnleitner, 2000).

P. pastoris cells can grow on a broad range of pH from 3.0 to 7.0, though the cell growth is not considerably affected within these pH values (Inan *et al.*, 1999).

Optimal pH for wild type *E. coli* growth ranges from 6.4 to 7.2 (Holt *et al.*, 1994). Çalık *et al.* (2006), investigated the effect of controlled and uncontrolled pH on benzaldehyde lyase production. Among the pH values ranging from 5.0 and 7.8, uncontrolled pH of 7.2 was determined as the most convenient condition for the activity of recombinant enzyme. Afterwards, recombinant benzaldehyde lyase was produced on molasses-based complex medium by *E.coli* BL21 strain with controlled pH condition at 7.2 (Kaya *et al.*, 2009; Çalık and Levent 2009-a,b).

For the production of thermostable GI, pH was adjusted to 5.0 for *P. pastoris* and 7.2 for *E. coli*. In bioreactor experiments of *E. coli*, pH was controlled and adjusted during the production using 5M KOH and 5M H_3PO_4.

2.4.4 Oxygen

The effect of oxygen on cell metabolism is very well-known. Since oxygen affects the metabolic pathways and metabolic fluxes inside the cell, it has diverse effects on protein production (Çalık *et al.*, 1999). Adaptation of *Saccharomyces cerevisiae* is determined by Furukawa *et al.* in 1983. During the growth of *Escherichia coli*, a clear relationship was observed between dissolved oxygen concentration and catabolic repression, inhibition and inducer repression of β-galactosidase (Pih *et al.*, 1988).

The effect of oxygen transfer rate was investigated by many researchers for production of several products by *E. coli* (Ryan *et al.*, 1989; Bhattacha and Dukey, 1997; Leon *et al.*, 2003; Çalık *et al.*, 2004). Kaya *et al.* (2009) investigated the oxygen effect on cell growth and product yield in a batch cultivation and reported the best condition as DO level of 40% air saturation . This value was further used in bioreactor experiments of *E. coli* developed by Çalık and Levent (2009-b) for benzaldehyde lyase production, Angardi and Çalık (2013) for glucose isomerase production, in the M.Sc. study of Taşpınar (2010) and Akdağ (2013).

2.5 Bioreactor Operation Modes

Fermentation of the cells can be performed in bioreactors operating in batch, semi-batch or continuous mode. Most bioprocesses are operated at batch or semi-batch modes due to low contamination risk, uncomplicated control of process, simplicity of set-up and operation and high product yields. However, continuous bioreactors have the advantage of consistent quality and flexibility of the system together with high product productivity (Dunford, 2012).

2.5.1 Batch

Microorganisms are added to the bioreactor after sterilization and the cooling of the medium is completed for cultivation. The medium contains substrates and nutrients at the beginning of the process. In order to control pH and foam, chemical agents might be added to the system. Since batch bioreactors require a particular time for cleaning sterilization, re-filling of the bioreactor, productivity of the process decreases. Moreover, cells are subjected to initial adaptation phase which is called lag-phase until they reach a desired concentration. To overcome these issues, cells are recycled from batch to batch in ethanol fermentation. In addition to that, microorganisms which have higher performance can reduce process time and size of fermentor and finally cost of operation (Wilkins and Atiyeh, 2012).

Microbial growth which is seen in batch mode can be divided into five phases and represented in Figure 2.16 (Liu, 2013):

1. Lag phase: It is used to describe the adaption time of the cells when they are inoculated to the new environment. Cells carry out the biosynthesis of new enzymes depending on the composition of the culture. Amount of the cell increases insignificantly during this stage. Age of inoculum, composition of the medium are important points which affect the duration of this phage.

2. Logarithmic or exponential growth phase: Following the adaptation period, cells proliferate rapidly since they begin to produce required metabolites to survive in the medium. The maximum rate of cell growth is seen in this phase. Cell mass within the media increase proportional with specific growth rate (μ [h^{-1}]: increase in cell mass per unit time) (equation 2.1) in this phase.

$$\frac{dCx}{dt} = \mu C_x \qquad\qquad 2.1$$

3. Deceleration phase: This phase represents the deceleration of growth after the exponential growth phase.

4. Stationary phase: Following the deceleration phase, cells reach stationary phase where the rate of cell formation is equal to the rate of cell death. Therefore; net cell growth rate is zero. Biosynthesis of some metabolites (e.g., hormones, antibodies) occurs in this phase.

5. Death phase: Due to the nutrient and substrate depletion and toxic by-product formation within the medium, rate of cell death increases.

2.5.2 Semi (Fed) –Batch

The beginning of the semi-batch operation overlaps with batch mode. After some time, a concentrated medium is fed to the system with an appropriate feeding rate which leads to the change of reaction volume gradually. This mode is chosen to minimize substrate inhibition therefore higher product yields are obtained in semi-batch mode than in batch-mode. In both batch and semi-batch operation modes, nothing is taken from the reactor during the bioprocess (Wilkins and Atiyeh, 2012).
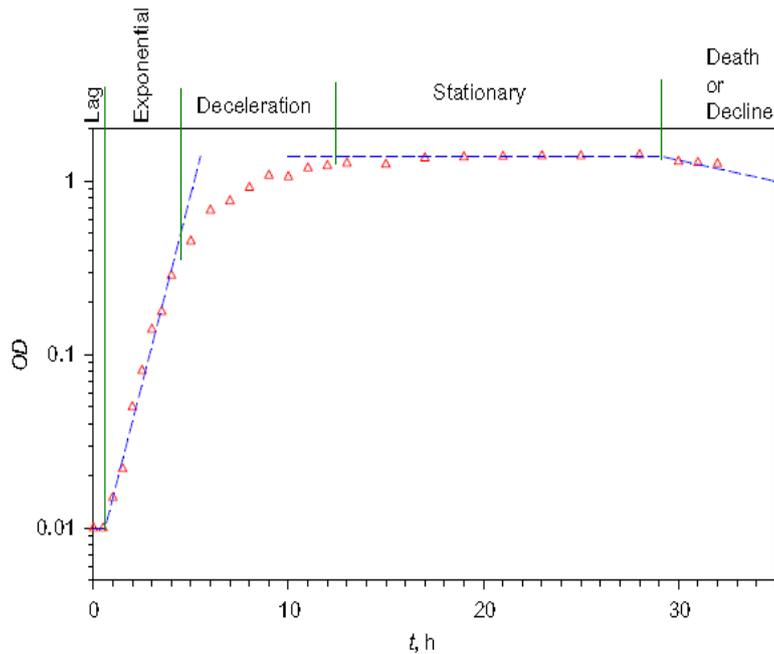
Figure 2.16 Semi-log plot for cell microbial cell growth in batch mode
(Liu, 2013).

Semi batch operation mode was used after World War I for the production of yeast biomass in which glucose concentration was increased gradually during fermentation to prevent cells from producing ethanol (White, 1954).

It is widely used in industrial fermentations when cultures are affected by substrate inhibition and catabolic repression (Tramper, 1994). It has been reported that limited glucose concentration within the medium prevents the Crabtree effect for *S. cerevisiae* and inhibition of cell growth (Kapelli, 1986).

Semi-batch operation is a dynamic process since it includes manipulation of limiting nutrient concentration by adjusting feed rates at a constant level or a pre-determined level usually followed by a final batch mode (Lim and Shin, 2013).

The substrate(s) which has a significant effect on cell growth and productivity must be determined by physiological and biochemical properties of the cells or even using trial and error method if necessary. Semi-batch mode for microbial processes is classified by Yamané and Shimizu (1984) and presented in Table 2.8. Semi-batch processes can be operated with or without feedback control. Constant, exponential,

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optimized semi-batch operations are subdivisions of processes without feedback control (Yamané and Shimizu, 1984).

Exponential feeding strategy is a technique that leads to cell growth at a pre-determined specific growth rate. *E. coli* strains both recombinant and wild type are subjected to this method for high cell density cultivation (Lee, 1996; Gregory and Turner, 1993; Yang and Epistein, 1992). Since acetate formation during *E. coli* fermentation could reduce cell growth and productivity, it is important to keep specific growth rate lower than the limit (0.1-0.35 h^{-1}) to prevent acetate formation. (Lee, 1996).

Constant feeding strategy is a technique where feed containing substrate and/or nutrients are fed to the system with a pre-determined constant value. Throughout the bioprocess, specific growth rate decreases as a result of increasing volume of the medium (Lee, 1996).

Optimized feeding refers to combination of exponential and constant feeding strategies to increase the product yield. Chen *et al.* (2012) developed an optimized feeding strategy for the production of glucosamine and N-acetylglucosamine by *E. coli*. After batch cultivation, exponential feeding with a specific growth rate of $\mu=0.20$ h^{-1} started until 10h of the process, then it was followed by constant feeding of concentrated glucose solution. 1.59-fold increase was obtained when compared to batch cultivation.

Table 2.8 Control of feed in semi-batch operations (Yamané and Shimizu, 1978)

Without feedback control
Intermittent addition
Constant rate
Exponentially increased rate
Optimized
Others
With feedback control
Indirect feedback control
Direct feedback control

Processes with feedback control are subdivided as direct where substrate concentration directly affects the rate of feeding and indirect feedback control where parameters like DO and pH affect the rate of feeding (Yamané and Shimizu, 1984).

2.5.3 Continuous

In continuous fermentation, low concentrations of substrate to prevent substrate toxicity and high cell concentrations are used. Continuous operation mode begins with a start-up phase which cells are propagated to desired concentrations for production and continues with steady-state phase in which the substrates and nutrients are fed to system at the same rate of withdrawal of the medium that contains cells. Therefore, the volume in the bioreactor is kept constant during the process. Cells in the removed medium can be separated with a module and recycled to the bioreactor to keep cell concentration at the desired value. Even though the continuous operation mode has the advantage of high productivity, it has lower final product concentrations than batch and semi-batch operation mode. The main issue of this mode is high risk of contamination and losing cells (wash-out) when high feed rates are used (Wilkins and Atiyeh, 2012).

2.6 Bioprocess Kinetics of Semi-Batch Operation Mode

In order to evaluate characteristics of the bioprocess, calculating parameters such as specific formation rate of the cells and product, consumption rate of substrate, yield and productivity is important. Cell and product formation and substrate consumption are determined with the help of the data taken during the bioprocess .

2.6.1 Mass Balance Equation for Cell Growth

Both in batch and semi-batch feeding processes, material balance for cell during the bioprocess is constructed as equation 2.2. Cell formation rate in bioprocess is usually termed as r_x [g L^{-1} h^{-1}], where V is liquid volume inside the bioreactor [L], C_X is cell concentration [g L^{-1}].

$$r_x V = \frac{d(C_x V)}{dt} \qquad\qquad 2.2$$

In equation 2.2, it was assumed that cell generation occurs as batch-wise and no volume change occurs due to sampling. Cell growth during bioprocess is characterized by specific growth rate which is termed as μ [h^{-1}]. It is one of the most considerable paramaters that reflects the chacartestic of the bioprocess. Since it is defined as increase in cell mas per unit time, equation 2.2 can be re-written as follows:

$$r_x = \mu C_x \qquad\qquad 2.3$$

By inserting equation 2.3 into 2.2, equation 2.4 can be derived as follows:

$$\mu C_x V = \frac{d(C_x V)}{dt} \qquad\qquad 2.4$$

In the study, the bioprocess is operated at semi-batch mode by pre-determined molasses feeding. Therefore, volume change as a result of feed substrate should be taken into account. The density of the liquid medium inside the bioreactor was assumed to be constant. From continuity equation for the semi-batch operation mode:

$$\frac{dV}{dt} = Q_{in} - Q_{out} \qquad\qquad 2.5$$

Volume change occurs due to substrate feeding. Since there is no withdrawal of the culture from bioreactor, Q_{out} can be taken as zero 0. Therefore, $Q_{in} = Q > 0$, where Q is volumetric flow rate [m^3 h^{-1}]. Hence, by inserting equation 2.5 into equation 2.4, equation 2.6 is obtained as follows:

$$\frac{dC_x}{dt} = \left(\mu - \frac{Q}{V}\right) C_x \qquad\qquad 2.6$$

Specific growth rate for bioprocess working at semi-batch mode can be obtained as follows by rearranging equation 2.6:

$$\mu = \frac{dC_x}{dt} \frac{1}{C_x} + \frac{Q}{v} \qquad\qquad 2.7$$

2.6.2 Mass Balance Equation for Substrate

In bioreactor system, mass balance equation for the substrate which is fed during the semi-batch bioprocess is constructed as follows:

$$Q_s C_{s_0} + r_s V = \frac{d(C_s V)}{dt} \qquad\qquad 2.8$$

r_s is substrate consumption rate, which is a function of q_s, specific substrate consumption rate [h^{-1}] and C_X cell concentration [g L^{-1}]. It is defined by a first order kinetic equation, where qs is the kinetic coefficient, as follows:

$$r_s = q_s C_x \qquad\qquad 2.9$$

By substituting equation 2.9 into 2.8, one can obtain equation 2.10 as follows:

$$Q_s C_{s_0} + q_s C_x V = C_s \frac{dV}{dt} + V \frac{dC_s}{dt} \qquad\qquad 2.10$$

By rearranging equation 2.10, specific substrate consumption qs is obtained as:

$$q_s = \frac{1}{C_x} \left(\frac{C_s}{V} Q + \frac{dC_s}{dt} - \frac{Q_s}{V} C_{s_o} \right) \qquad\qquad 2.11$$

Since in semi-batch operation mode, substrate is fed to the system in accordance with cells' demands to prevent substrate accumulation, the system can be

considered at quasi-steady state. Hence, dC_S/dt can be taken as zero. r_S can also be defined in terms of yield (specific) coefficient $Y_{X/S}$:

$$-r_s = (r_x / Y_{x/s})$$ 2.12

By inserting equation 2.12 into 2.2, one can obtain equation 2.13 as follows:

$$Q_s C_{s_0} - \frac{r_x V}{Y_{x/s}} = C_s \frac{dV}{dt}$$ 2.13

If the terms $r_X = \mu C_X$ and $dV/dt = Q_S$ are inserted into previous equation:

$$Q_s C_{s_0} - \frac{\mu C_x V}{Y_{x/s}} = C_s Q_s$$ 2.14

With the solution of differential equation 2.2 together with 2.3,

$$C_x V = C_{x_0} V_0 e^{\mu t}$$ 2.15

By inserting the equation 2.15 into equation 2.14, equation for volumetric feed rate of substrate Q_S can be derived as:

$$Q_s = \frac{\mu C_{x_0} V_0}{Y_{x/s}(C_{s_0} - C_s)} e^{\mu t}$$ 2.16

2.6.3 Overall Yield Coefficient

Yield (selectivity) coefficient can be determined as the ratio of molecules produced to the quantity of molecules consumed during the reaction. Equation for cell yield on substrate is presented in equation 2.17, as follows:

$$Y_{x/s} = \frac{r_x}{-r_s} = \left(\frac{dC_x/dt}{-dC_s/dt} \right)$$ 2.17

If the yield coefficients are calculated for a finite period of time, overall yield coefficients for cell and product on substrate can be defined as:

$$\bar{Y}_{X/S} = \frac{r_x}{-r_s} = \frac{\Delta C_x / \Delta t}{-\Delta C_s / \Delta t} \qquad\qquad 2.18$$

Yields coefficients that are often used to evaluate characteristics of bioprocesses are presented in Table 2.9.

Table 2.9 Yields coefficients of bioprocesses

Symbol	Definition
$Y_{X/S}$	Mass of cell produced per unit mass of substrate consumed
$Y_{P/S}$	Mass of product produced per unit mass of substrate conssumed
$Y_{P/X}$	Mass of product produced per unit mass of cell produced
$Y_{X/O}$	Mass of cell produced per unit mass of oxygen consumed
$Y_{S/O}$	Mass of substrate consumed per unit mass of oxygen consumed
$Y_{P/O}$	Mass of product produced per unit mass of oxygen consumed

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals used in the study were analytical grade and purchased from Sigma-Aldrich Co., and Merck & Co., Inc.

3.2 Buffers and Stock Solutions

All the buffers and stock solutions used in this study are presented together with their formulations in Appendix A. Sterilization of prepared solutions were done by autoclaving at 121 ^{o}C for 20 minutes or by filter sterilization through filters having 0.25/0.45 μm pore size (Sartorius AG, Germany).

3.3 The Microorganisms, Plasmids and Maintenance of Microorganisms

Wild type strain of *Pichia pastoris* X-33 was purchased from Invitrogen. *E. coli* TOP10 cells carrying pRSETA shuttle vector containing *xylA* gene was named as *E. coli* TOP10 pPICZ-A::xyl$_{int}$ (Ata, 2012) and used for the isolation of pPICZ-A shuttle vector containing *xylA* gene. The recombinant *P. pastoris* X33 strain which was transfected with pPICZ-A shuttle vector isolated from *E. coli* TOP10 pPICZ-A::xyl$_{int}$ and producing GI intracellularly was named as *P. pastoris* S18. The recombinant *E. coli* BL21 (DE3) pLysS carrying pRSETA and producing GI

intracellularly was named as *E. coli* V11 (Angardi and Çalık, 2013). Strains and plasmids used in this study are stated in Table 3.1. Microbanks were prepared for long-term storage of the cells. Young cell colonies were inoculated into cyrovials (PRO-LAB) containing cyropreservative fluid, for this purpose. After gently mixing the cells and fluid for 5 seconds, it was waited until the absorption of the cells onto the porous beads for 1-2 minutes. Then, cyropreservative fluid was removed and prepared microbanks were kept at -55°C.

3.4 Growth Media

The sterilization of the growth media or components used in the study was done by autoclaving at 121 $^{\circ}$C for 20 minutes or by filter sterilization through filters having 0.25/0.45 μm pore size (Sartorius AG, Germany). After sterilization, appropriate antibiotics were added to the media if needed (ampicillin resistance in pRSET plasmid; chloramphenicol resistance in BL21 (DE3) pLysS cells; Zeocin resistance in pPICZ-A plasmid). The concentrations of the antibiotics are presented Table 3.4.

Table 3.1 Strains and plasmids used in this study.

Genus	Species	Strain	Genotype/Plasmid	Source
Pichia	*pastoris*	X33	Wild type	Invitrogen
Pichia	*pastoris*	S18	pPICZ-A::*xylA*$_{int}$	In this study
Escherichia	*coli*	TOP10	pPICZ-A::*xylA*$_{int}$	(Ata, 2012)
Escherichia	*coli*	V11	pRSETA::*xylA*$_{int}$	(Angardi and Çalık, 2013)

3.4.1 Solid Media and Growth Conditions

Recombinant *P. pastoris* S18 cells were taken from microbank which was kept at -55°C, cultivated on YPD agar containing 0.15 g L^{-1} Zeocin and incubated at 30°C for 48-60 hours. Wild type *P. pastoris* X33 cells were cultivated on YPD agar media without addition of antibiotics. Composition of YPD agar medium is presented in Table 3.2.

Recombinant *E. coli* TOP10 cells carrying pPICZ-A::*xyl*$_{int}$ gene were taken from microbank and transferred to low-salt LB agar containing 0.025 g L^{-1} Zeocin and incubated at 37°C for 24 hours. Recombinant *E. coli* V11 cells were incubated on low-salt LB agar media containing 0.1 g L^{-1} ampicillin together with 0.035 g L^{-1} chloramphenicol. Composition of low-salt LB agar is presented in Table 3.3.

Table 3.2 Composition of YPD agar medium.

Compound	Concentration (g L^{-1})
Yeast extract	10
Peptone	20
Glucose*	20
Agar	20

*Glucose was filter sterilized and added to the medium after autoclave.

Table 3.3 Composition of low-salt LB agar medium.

Compound	Concentration (g L^{-1})
Soytryptone	10
Yeast extract	5
NaCl	5
Agar	15

Table 3.4 Anbitiotic concentration of the media for recombinant *E. coli* and *P. pastoris* strains.

Antibiotic	Microorganism	Concentration (g L^{-1})
Ampicillin	*E. coli* V11 pRSETA::*xylA*$_{int}$	0.1
Chloramphenicol	*E. coli* V11 pRSETA::*xylA*$_{int}$	0.035
Zeocin	*E. coli* TOP10 pPICZ-A::*xylA*$_{int}$	0.025
Zeocin	*P. pastoris* S18	0.15

3.4.2 Precultivation Media

Recombinant *P. pastoris* S18 and *E. coli V11* cells which were incubated on solid media were transferred to precultivation media.

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3.4.2.1 Precultivation Medium for *P. pastoris* S18

Precultivation of recombinant *P. pastoris* S18 was performed in 250 ml baffled air-filtered shake bioreactors containing 50 ml BMGY (buffered glycerol complex medium) in an orbital shaking incubator (B. Braun, Certomat BS-1) with agitation rate of N= 225 rpm and temperature of T=30 ^{o}C. Composition of BMGY medium is presented in Table 3.5.

3.4.2.2 Precultivation Medium for *E. coli*

Recombinant *E. coli* V11 precultivation was performed in 150 ml baffled air-filtered shake bioreactors containing 33 ml low-salt LB medium. Growth conditions of *E. coli* cells were set to agitation rate of N=200 rpm and temperature of T=37 ^{o}C in orbital shaking incubator for 12h. Composition of low-salt LB medium is

presented in Table 3.6. Appropriate concentrations of antibiotics were added according to the amounts presented in Table 3.4

Table 3.5 Composition of BMGY precultivation medium for recombinant *P. pastoris* S18

Compound	Concentration (g L^{-1})
Yeast extract	10
Peptone	20
Potassium phosphate buffer (pH=6.0)	0.1 M
Yeast nitrogen base (YNB) (w/o amino acids)	13.4
Biotin *	4 x 10-5
Glycerol	10
Chloramphenicol (34 mg ml^{-1})	1ml L^{-1}

*Biotin and chloramphenicol were added to the medium after autoclave.

Table 3.6 Composition of low-salt LB precultivation medium for recombinant *E. coli* V11

Compound	Concentration (g L^{-1})
Soytryptone	10
Yeast extract	5
NaCl	5
Ampicillin*	0.1
Chloramphenicol*	0.035

* Ampicillin and chloramphenicol were added to the medium after autoclave.

3.4.3 Production Media

3.4.3.1. Production Medium for Air-Filtered Shake Bioreactor Experiments of *P. pastoris* S18

Recombinant *P. pastoris* cells were proliferated in precultivation medium until the cell concentration reached to OD_{600} of 2-6. Afterwards, cells were harvested, centrifuged and resuspended into production medium (Table 3.7). Cell concentration within production medium was adjusted to $OD_{600}=1$.

Nitrogen source ammonium sulfate was added to the medium according to the carbon nitrogen ratio (C/N) of 4.57 determined by Jungo *et al.* (2006) (Table 3.9).

Table 3.7 Production medium for recombinant *P. pastoris* S18 cells (Jungo *et al.,* 2006)

Compound	Concentration (g L^{-1})
Methanol	1 mL
Sorbitol	*
Ammonium sulfate	**
Potassium phosphate buffer pH=6	0.1 M
$MgSO_4.7H_2O$	14.9
$CaSO_4.2H_2O$	1.17
Chloramphenicol	1 mL
PTM1	4.35 mL

Composition of PTM1 trace salt solution is presented in Table 3.8 (Sibirny *et al.*, 1990).

Table 3.8 Composition of PTM1 trace salt solution (Sibirny *et al*., 1990).

Compound	Concentration (g L^{-1})
$CuSO_4.5H_2O$	0.6
NaI	0.008
$MnSO_4.H_2O$	0.3
$Na_2MoO_4.2H_2O$	0.02
H_3BO_3	0.002
$ZnCl_2$	2
$FeSO_4.7H_2O$	6.5
$CoCl_2.6H_2O$	0.09
H_2SO_4	0.5
Biotin	0.02

Table 3.9 Concentration of nitrogen and carbon sources of the shake bioreactor production medium for *P. pastoris* S18

Sorbitol concentration (g L^{-1})*	Ammonium sulfate concentration (g L^{-1})**
10	4.76
20	14.28
30	23.8

3.4.3.2 Production Media for *E. coli* V11

The recombinant *E. coli* cells which were subjected to precultivation procedure, then transferred into production medium at the inoculation ratio of 1:10. Production media for air- filtered shake bioreactor experiments and pilot scale bioreactor experiments are presented in Table 3.10 and Table 3.11, respectively.

Table 3.10 Composition of production medium for air- filtered shake bioreactor experiments of *E. coli* V11

Compound	Medium Composition (g L^{-1})			
	Glucose+Fructose	Sucrose	Molasses	Hydrolyzed Molasses
Molasses	-	-	30	30
Glucose	8	-	-	-
Fructose	8	-	-	-
Sucrose	-	16	-	-
Na_2HPO_4	6.7	6.7	6.7	6.7
KH_2PO_4	3.1	3.1	3.1	3.1
$(NH_4)_2HPO_4$	5.0	5.0	5.0	5.0
NaCl	0.5	0.5	0.5	0.5
$MgSO_4.7H_2O$	0.5	0.5	0.5	0.5
$ZnSO_4.7H_2O$	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}	0.2×10^{-5}
$MnSO_4.7H_2O$	0.1	0.1	0.1	0.1
Ampicillin	0.1	0.1	0.1	0.1
Chloramphenicol	0.035	0.035	0.035	0.035

Table 3.11 Composition of production medium for pilot scale experiments of *E. coli* V11

Compound	Concentration (g L^{-1})
Molasses	30
Na_2HPO_4	6.7
KH_2PO_4	3.1
$(NH_4)_2HPO_4$	5.0
NaCl	0.5
$MgSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.2×10^{-5}
$MnSO_4.7H_2O$	0.1
Ampicillin	0.1
Chloramphenicol	0.035

3.4.3.2.1 Acid Hydrolysis of Molasses

Molasses used in the study was obtained from Ankara Beet Sugar Factory (Turkey).

Since *E. coli* V11 could not utilize sucrose as a carbon source, molasses was subjected to acid hydrolysis pretreatment (Çalık *et al.*, 2003) to obtain 1:1 mixture of invert sugars glucose and fructose from the degradation of sucrose. Pretreatment steps are presented below:

1. 200 g molasses was diluted with ultra pure water to obtain final volume of 500 ml solution.
2. Diluted solution was centrifuged at 6000 g at $+4^{o}C$ for 20 minutes in order to remove impurities and insoluble compounds.
3. Molasses solution was pretreated with 37% HCl at room temperature until the pH of the solution was lowered to 1.8.
4. Solution was incubated in water bath at $90^{o}C$ subsequently and acid hydrolysis pretreatment was carried out for 3 hours.
5. After pretreatment procedure, pH of the solution was adjusted to 7.2 with the addition of 5M KOH for the growth of *E. coli*.

3.5 Genetic Engineering Techniques

3.5.1 Plasmid DNA Isolation from *E. coli* TOP10 Cells

E. coli TOP10 cells carrying pPICZ-A::*xylA*$_{int}$ were subjected to plasmid isolation procedure according to Alkaline Lysis Midiprep Method reported by Sambrook and Russell (2001). For this purpose;

1. Recombinant *E. coli* TOP10 cells were proliferated on LSLB agar medium containing appropriate antibiotic, Zeocin.
2. A single colony was selected from agar plate and transferred into 10 ml LSLB broth for proliferation of the cells carrying plasmid and incubated in orbital shaking incubator at $37^{o}C$.

3. Medium was transferred to 15 ml sterile falcon tube after overnight incubation, centrifuged 10 minutes at $4^{\circ}C$, 2000g.

4. Supernatant was removed and cell pellet was resuspended in 200 µl ice-cold Alkaline Lysis Solution I. Mixture was vigorously vortexed to assure complete dispersion of the cells in solution I and the content was transferred to 1.5 ml eppendorf tube.

5. 400 µl Alkaline Lysis Solution II was added to the mixture. Solution and the content were mixed by inverting the tube for 5-6 times. Subsequently, it was stored on ice for 3-5 minutes.

6. After the centrifugation of the content at $4^{\circ}C$, 16100g for 5 minutes, 600 µl was taken from supernatant was transferred to a fresh eppendorf tube.

7. An equal volume of phenol: chloroform was added. After mixing by vortexing, the solution was centrifuged at $4^{\circ}C$ 16100 g for 2 minutes.

8. Aqueous upper phase was transferred to a fresh eppendorf. 600µl isopropanol was added to the content and it was mixed by vortexing again. Afterwards, mixture was stored at room temperature for 2 minutes.

9. Thereafter, the content was centrifuged at 16100g, room temperature for 5 minutes.

10. Supernatant was removed. In order to drain away the remaining fluid, the tube was kept in inverted position for 4-5 minutes.

11. 1 ml of 70% ethanol was added to the tube.

12. After centrifugation of the contents at room temperature for 5 minutes at 16100 g, supernatant was removed carefully.

13. Remaining ethanol beads on the tube walls were removed by evaporation at room temperature.

14. When the ethanol was removed completely, 100 µl of TE buffer (pH: 8.0) containing 20 µg ml^{-1} RNase A was added to the pellet and the content mixed gently.

15. For further use of isolated plasmid DNA, the tube was stored at $-20^{\circ}C$.

3.5.2 Agarose Gel Electrophoresis

DNA fragments were visualized, sized and quantified by agarose gel electrophoresis. Agarose gel was prepared by dissolving 1% (w/v) g agarose in 1XTBE buffer, heating the solution on a hot plate until boiling point. When it was cooled down to approximately 45-50°C, 2.5 µl of ethidium bromide (Sigma-10 mg ml^{-1}) was added to be able to visualize DNA fragments. Thereafter, the gel was poured into a tray and comb was inserted immediately before the solidification of the gel. After approximately 10-15 minutes, the comb was removed from solidified gel and it was transferred to a tank containing 1XTBE buffer. DNA samples mixed with 6X loading dye which at a final volume of 1:5 were loaded to the wells together with DNA ladder (λ DNA/HindIII marker) to estimate the size of the fragments. Electrophoresis was applied for 45-55 minutes at 90V until the fragments were separated from each other. Finally, visualization of the strands was done by UV transilluminator (UVP BioImaging System, and Hamamatsu Digital CCD Camera) at 302 nm using UVP Bio Imaging Systems Labworks Image Acquisition and Analysis Software.

3.5.3. Restriction Endonuclease Digestion of Plasmid DNA

pPICZ-A::*xylA*$_{int}$ was linearized for transfection of *P. pastoris* X33. For this purpose; the plasmid DNA was incubated for 2 hours with one of the restriction enzymes (*SacI*) cutting in multiple cloning site of the plasmid. After digestion, the reaction was terminated by heat inactivation of the enzyme, keeping the mixture at 65°C for 20 minutes. Verification of the digestion was performed by agarose gel electrophoresis. Amounts of compounds in reaction mixture are presented in Table 3.12.

Table 3.12 Composition of restriction digestion mixture

Compound	Amount
pPICZ-A::$xylA_{int}$	30-50 µg
10X *Sac* I Buffer	5 µl
SacI (10 U µl^{-1})	2 µl
H_2O	Up to50 µl

3.5.3.1 Purification of Digested Plasmid DNA

After restriction endonuclease digestion, linearized plasmid was purified for further procedures. First, equal volume of phenol: chloroform was added to reaction mixture, mixed and centrifuged at maximum speed for 1 minute at room temperature. After centrifugation, aqueous phase was transferred to a fresh eppendorf. 1/10 volume of 3M Na-acetate and 2.5 volume of 100 % ethanol were added. The mixture was centrifuged under the same conditions as before. Supernatant was decanted and the pellet was washed by 80% ethanol and centrifuged. The supernatant was decanted again and the pellet was dissolved in sterile water.

3.5.4 Transfection of *Pichia pastoris* X33

Transfection of *P. pastoris* X33 with pPICZ-A::$xylA_{int}$ vector was achieved according to LiCl method (Invitrogen Cat. no. K1740-01, 2010):

1. *P. pastoris* X33 cells were inoculated on YPD agar without any antibiotics, first. Agar plates were incubated at 30°C for 48 hours.
2. A single colony which was selected from agar plate was transferred to 50 ml YPD broth again without any antibiotics and incubated in orbital shaking incubator at T= 30°C, N= 200 rpm until OD_{600} of the cells reached the value between 0.8 and 1 (approximately 10^8 cells ml^{-1}; t=12-16 h).
3. Afterwards, the cells were harvested and centrifuged at 1500g at room temperature for 10 minutes.

4. Supernatant was decanted and cell pellet was washed with 25 ml sterile water.

5. Centrifugation at 1500g at room temperature for 10 minutes was carried out and water was separated from the cells by discarding supernatant.

6. The cell pellet was resuspended in 1 ml of filter sterilized 100 mM LiCl and the content was transferred to an eppendorf tube.

7. The cells were precipitated by centrifuge at maximum speed for 15 seconds.

8. LiCl was removed with a micropipette and the cell pellet was resuspended in 400 μl of filter sterilized 100 mM LiCl.

9. 50 μl of the cell suspension was allocated into 1.5 ml eppendorf tubes for each transformation and used immediately. The suspension was centrifuged at maximum speed for 15 seconds and LiCl was removed with a micropipette.

10. 240 μl of 50% PEG, 36 μl of 1 M LiCl, 25 μl of 2 mg ml^{-1} single-stranded DNA, 50–100 μg of linearized pPICZ-A::$xylA_{int}$ plasmid DNA in 50 μl sterile water was added onto the cell pellet in the order given. Linearization of the plasmid is presented in section 3.5.3. Each tube was vortexed until the cell pellet and the reagents were mixed thoroughly and incubated at T=30°C for 30 minutes without shaking.

11. Following 30 minutes incubation, heat shock was applied to the tube by holding it in water bath at T=42°C for 20-25 minutes.

12. Thereafter, the tube was centrifuged at 6000-8000 rpm and reagent solution used for transformation was removed from cell pellet by a micropipette.

13. The cell pellet was resuspended in 1 ml of YPD (Table 3.13) and incubated at 30°C for 2 hours.

14. Finally, 25-100 μl was taken from YPD containing cells and transferred to 150 mg ml^{-1} Zeocin added YPD plates and incubated for 2-3 days at 30°C.

Table 3.13 Composition of YPD broth medium

Compound	Concentration (g L^{-1})
Yeast extract	10
Peptone	20
Glucose*	20

3.5.5 Microorganism Selection

After transfection of *P. pastoris*, the accuracy of the procedure was confirmed by PCR which was done using isolated genomic DNA. The strains carrying the recombinant plasmid pPICZ-A::*xylA*$_{int}$ were determined by evaluating agarose gel electrophoresis of PCR products. After the true colony was selected based on PCR results, activity assay was carried out to see intracellular GI production capacity of selected transformant.

3.5.5.1 Isolation of Genomic DNA from Yeast

Genomic DNA isolation was performed to validate transfection of *P. pastoris*. Cells that were incubated on YPD plates for 2-3 days after transfection were directly used for genomic DNA isolation. The method developed by Amberg *et al.* (2000) was used with a few modifications. After incubation, a single colony was transferred to an eppendorf tube containing 200 µl of yeast lysis buffer. Thereafter, 200 µl of phenol: chloroform: isoamylalcohol (25:24:1) added to the tube and the mixture was vortexed for 4 minutes following the addition of 0.3 g acid-washed glass beads. Later, 200 µl of TE Buffer (pH: 8.0) was added and the mixture was centrifuged at 4000 g for 2 minutes at room temperature. Aqueous phase was separated and transferred to a fresh eppendorf. 1 ml of 100% ethanol was added to aqueous phase. After mixing by inversion, it was centrifuged for 2 minutes at maximum speed. Supernatant was decanted and 400 µl of TE buffer and 10 µl of 10 mg ml^{-1} RNase A was added to the pellet. For 5 minutes, the mixture was kept in 37°C water bath.

Thereafter, it was treated with 1 ml of 100% ethanol and 14 µl of 3 M sodium acetate. After centrifugation at maximum speed for 2 minutes, supernatant was decanted and pellet which was consisting of DNA was air dried and dissolved in 50 µl of TE buffer (pH: 8.0). Isolated DNA was used in PCR and stored at -20°C for further use.

3.5.5.2 Confirmation of Transfection by PCR

After transfection of *P. pastoris*, genomic DNA of colonies were isolated and used as templates in PCR. Since integration of *AOX1* and GI region on pPICZ-A::*xylA*$_{int}$ plasmid into the genome of *P. pastoris* was aimed for in the transfection, these two regions were targeted for PCR validation. Primers specific to the sequences of *xylA* and *AOX1* were designed by Ata (2012) were used in PCR. Two sets of PCR reaction was perfomed by a thermocycler (Techgene, Flexigene) for *xylA* and *AOX1* separately. Primers used in the study, PCR reaction mixture and conditions are presented in Table 3.14, Table 3.15 and Table 3.16, respectively.

Table 3.14 Primers used for the amplification of *xylA* and *AOX1*. Underlined sequences are restriction enzyme recognition sites.

	Name	Sequence	Target Sequence In Genome of *P. pastoris*
1st set	-GI Forward Primer	5'C<u>TTCGAA</u>ATGTACGAGCCCAAACCA GAGCACAGG-3'	*xylA*
	-GI Reverse Primer	5'-CG<u>TCTAGA</u>TCACCCCCGCACCCCCA GGAAGTACTC-3'	*xylA*
2nd set	-*AOX1* Forward Primer	5'-GACTGGTTCCAATTGACAAGC-3'	*AOX1*
	-*AOX1* Reverse Primer	5'-GCAAATGGCATTCTGACATCC-3'	*AOX1*

Table 3.15 Composition of PCR reaction mixture.

Compound	Amount
10X PCR Buffer (-Mg^{++})	5 μl
dNTP (2 mM)	10 μl
MgSO$_4$ (25 mM)	6 μl
Forward Primer (5 μM)	5 μl
Reverse Primer (5 μM)	5 μl
Template DNA (0.01-1 μg)	0.5-5 μl
Pfu DNA polymerase (2.5 U μl^{-1})	0.5 μl
H$_2$O	50 μl (Total volume)

Table 3.16 PCR conditions for the amplification of *xylA* and *AOX1* region.

Number of cycles	Temperature	Time (min)
1 cycle	94°C	3
1 cycle	94°C	1
	44°C	1
	72°C	3
29 cycle	94°C	1
	50°C	1
	72°C	3
1 cycle	72°C	10
	4°C	∞

3.6 Recombinant Protein Production

Recombinant thermostable GI production was carried out by *P. pastoris* carrying pPICZ-A::*xyl*A$_{int}$ in laboratory scale air- filtered shake bioreactors and by *E. coli* carrying pRSETA::*xylA*$_{int}$ in both shake bioreactors and pilot scale bioreactors.

3.6.1 Air-Filtered Shake Bioreactor Experiments of *P. pastoris*

In air-filtered shake bioreactor experiments of *P. pastoris*, different concentrations of a co-carbon source were examined. Considering its non-repressing effects on *AOX1* promoter and positive effects on cell growth (Sreekrishna *et al.*, 1997), sorbitol was selected as a co-carbon source. Using orbital shaking incubators at N=225 rpm at 30°C, cells were proliferated in precultivation medium BMGY until OD$_{600}$ of 2-6. Thereafter, they were transferred to production medium containing C_{S0}=10 g L^{-1}, C_{S0}=30 g L^{-1} or C_{S0}=50 g L^{-1} sorbitol concentration (Table 3.7). Cells were harvested by centrifuge at 1500g at 4°C for 10 minutes and diluted to have OD$_{600}$=1 in production medium initially. Batch production of recombinant protein was carried out in 250 ml baffled air-filtered shake bioreactors containing 50 ml production medium. Using orbital shaking incubator with the same conditions as precultivation, recombinant protein production was carried out for 54h and triggered by induction of *AOX1* promoter by methanol (1%, v/v) every 24h.

3.6.2 Air-Filtered Shake Bioreactor Experiments of *E .coli*

In air-filtered shake bioreactor experiments of *E. coli*, cells were inoculated in production medium (Table 3.10) containing C_{M0}=30 g L^{-1} unhydrolyzed molasses, C_{M0}=30 g L^{-1} hydrolyzed molasses, C_{S0}=16 g L^{-1} sucrose, C_{G0}=8 g L^{-1} glucose+ C_{F0}=8 g L^{-1} fructose to examine the effect of different carbon sources on cell growth of *E. coli BL21* (DE3) carrying pRSETA::*xylA*$_{int}$. For this purpose, cells that were proliferated in LSLB precultivation medium for 12 hours were transferred to production medium with the inoculation ratio of 1:10. Shake bioreactor experiments were carried out in orbital shaking incubators for 12 hours using the same conditions as precultivation. At t=4h of the production, IPTG was added to the fermentation media containing glucose+ fructose and sucrose as carbon sources as final concentration of 1mM for induction T7 expression system which was under the control of *lacUV5* promoter (Angardi and Çalık, 2013). Since molasses containing media contains galactose, they did not require IPTG as inducer. Composition of

production medium for air- filtered shake bioreactor experiments of *E. coli* V11 is presented in Table 3.10

3.6.3 Pilot Scale Bioreactor Experiments of *E. coli*

At the beginning of the pilot scale bioreactor experiments, cells grown on LSLB agar for 24 hours were transferred to LSLB precultivation medium. After the incubation in precultivation medium for 12 hours, cells were transferred to bioreactor with the inoculation ratio of 1:10. The production medium for pilot scale bioreactor experiments is presented in Table 3.11.

3.6.3.1. Pilot Scale Bioreactor

Recombinant thermostable glucose isomerase production was conducted in a 3L pilot scale fermentor (Braun CT2-2) having a working volume of 1L. In bioreactor experiments; temperature, dissolved O_2, pH, stirring rate, foam and feed inlet rate was controlled with the units present in fermentor (Figure 3.1) Sterilization and temperature control of bioreactor was conducted by external cooler, steam generator and a jacket. Agitation was performed by two Rushton turbines containing four blades and a sparger. The air supplement into the bioreactor was provided by a compressor. Acid, base, antifoam and feed solutions were fed to the system through the inlet ports.
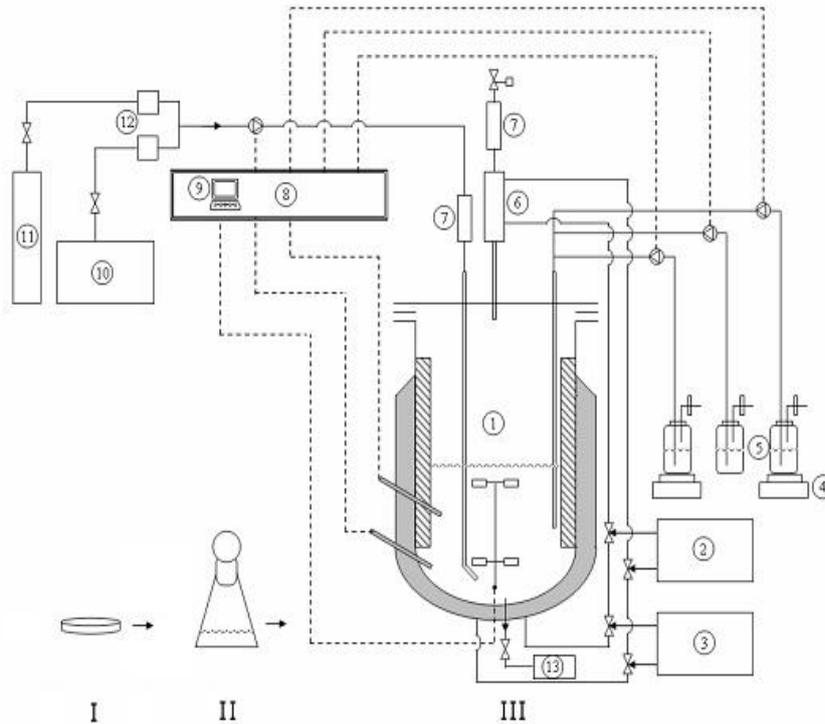
Figure 3.1 Schematic representation of pilot scale bioreactor system together with precultivation steps. I. Solid medium containing inoculated cells from microbank. II. Precultivation medium II. Pilot scale bioreactor system. (1) Bio-reaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2 tank (12) Digital mass flow controllers (13) Sampling bottle (Çelik, 2008)

3.6.3.2 Operation Parameters of Bioreactor Experiments

Temperature of the bioprocess was kept constant at $37^{o}C$ by an external cooler and a steam generator. Dissolved O_2 concentration in fermentation medium was kept at 40% air saturation by adjustment of air flow rate which was operating in cascade mode. 5M KOH and 5M H_3PO_4 were used in order to keep pH constant at 7.2. Agitation rate was N=750 rpm to provide effective transfer of oxygen, heat and medium compounds in the fermentation medium. Minor amounts of 10% antifoam solution were added to remove excess foam when necessary.

In all feeding strategy development experiments, molasses concentration in bioreactor was C_{M0}=30 g L^{-1} initially. Until t=7h of the bioprocess, the same batch conditions were used for all strategies. After t=7h of the bioprocess, continuous or pulse feeding of molasses was applied. Following the pulse feeding, continuous feeding of molasses was started in semi-batch operation. All continuous and pulse feeds contained (NH$_4$)$_2$HPO$_4$ as nitrogen and phosphorus source. Ampicillin and chloramphenicol were added to the feeding media before feeding cells into the bioreactor in concentrations of 0.2 g L^{-1} and 0.07 g L^{-1}, respectively. Pulse and continuous feeds contained antibiotics twice of their regular concentrations. Volumetric feed rate of the substrate (Q$_S$) (equation 3.1), was derived from equation 2.16 in Section 2.6.2 by assuming C_{S0}>>C_S in the bioreactor. In all bioreactor experiments, pre-determined volumetric feed rate of molasses was calculated according to equation 3.1:

$$Q_S(t) = \frac{Cx_0 V_0 \mu_0}{Cs_0 Y_{X/S}} exp(\mu_0 t)$$ 3.1

Where, μ_0 is pre-determined specific growth rate [h^{-1}], Vo is initial reaction volume [L], C_{X0} is cell concentration reached before the beginning of continuous feeding [g L^{-1}], C_{S0} is substrate concentration in feed stock [g L^{-1}], $Y_{X/S}$ is cell yield on substrate [g g^{-1}]. Parameters of equation 3.1 are presented in Table 3.17.

Table 3.17 Parameters of equation 3.1 for molasses semi-batch operation.

Paramater	Value
Vo [L]	1
C_{S0} [g L^{-1}]	250
$Y_{X/S}$ [g g^{-1}]*	0.32

* Çalık and Levent (2009-b).

3.6.3.3 Feeding Strategies Applied in Bioreactor Experiments

In all bioreactor experiments, batch production was carried out until t=7h of bioprocess.

- ❖ In first bioreactor experiment, M-0.05, after t=7h of the process, continuous feeding of molasses containing $(NH_4)_2HPO_4$ as nitrogen and phosphorus source was started. Exponential feeding rate was calculated based on the specific growth of μ_0=0.05 h^{-1}.

- ❖ In second bioreactor experiment, M-0.1, after t=7h of the process, continuous feeding of molasses containing $(NH_4)_2HPO_4$ was started. Exponential feeding rate was calculated based on the specific growth of μ_0=0.1 h^{-1}.

- ❖ In third bioreactor experiment, PM-0.05, after t=7h of the process, a molasses pulse containing $(NH_4)_2HPO_4$ was given to the bioreactor to increase glucose concentration in the fermentation medium to its initial value. Thereafter, at t=10h of the process, continuous feeding of molasses containing $(NH_4)_2HPO_4$ was started based on the specific rate of μ_0=0.05 h^{-1}.

- ❖ In fourth bioreactor experiment, 2PM-0.05, second molasses pulse containing $(NH_4)_2HPO_4$ was given after first pulse of PM-0.05 at t=10h of the process to increase glucose concentration in the medium to its initial value. Then, continuous feeding of molasses containing $(NH_4)_2HPO_4$ was started based on the specific rate of μ_0=0.05 h^{-1} at t=11h hour of the process.

Applied feeding strategies are briefly explained in Table 3.18.

Table 3.18 Explanation of applied bioreactor strategies for recombinant thermostable GI production

No. of Bioreactor Experiment	Abbreviation	Specific Growth Rate (μ_o)	Total Cultivation Time (h)	Strategy Applied
1	M-0.05	0.05	34	Semi-batch operation started at t=7h.
2	M-0.1	0.1	33	Semi-batch operation started at t=7h.
3	PM-0.05	0.05	32	Molasses pulse feeding started at t=7h, Semi-batch operation started at t=10h.
4	2PM-0.05	0.05	30	1st molasses pulse feeding started at the t=7h, 2nd molasses pulse feeding started at t=10h, Semi-batch operation started at t=11h.

3.7 Analysis

In order to determine cell, glucose, fructose, aminoacid concentrations fermentation medium was taken from bioprocess every two hours. Afterwards, cells were harvested by centrifugation at 16100g at 4°C for 10 minutes. Supernatant was removed from cell pellet and transferred to fresh eppendorf tubes. Cell pellet was used to determine glucose isomerase activity. Glucose, fructose and organic acid concentrations were analyzed from filtered supernatant.

3.7.1 Cell Concentration

Cell concentration was determined as dry cell weight per liter by using UV-Vis spectrophotometer (Thermo Spectronic, Heiλosα) at 600 nm wavelength. In order to stay in the ranges of accurate measurement of spectrophometer, samples taken from fermentation medium were diluted with distilled H_2O to have absorbance values between 0.1 and 0.8. The equations obtained from calibration curves for dry cell weight of *P. pastoris* and *E. coli,* are given respectively.

$$Cx = \frac{OD_{600}}{2.8782} x DilutionFactor \qquad\qquad 3.2$$

$$Cx = OD_{600} x \, 2.75 x DilutionFactor \qquad\qquad 3.3$$

3.7.2 Enzyme Activity

One unit GI activity was defined as 1 μmol D-fructose converted from D-glucose per minute at 80°C. Therefore, glucose isomerase activity determination was based on detecting the converted concentration of D-glucose to D-fructose. For this purpose, 1 ml of sample was taken from fermentation broth was transferred to eppendorf tubes. Cells were harvested at 16100 g 4°C for 10 minutes. Supernatant was removed and the cell pellet dissolved in 970 μl of 0.1 M potassium phosphate buffer. Since GI was produced intracellularly, glass beads (diameter of ˜ 0.50 mm) were added on cell suspension inside the tube and the cell wall was disrupted by agitator bead mill (Retsch, MM 200) operating at f=10 s^{-1} for 10 minutes. 50 μl was taken and transferred to a fresh eppendorf tube. 50 μl freshly prepared activity buffer containing 0.4 M D-glucose and 10 mM MnCl$_2$ in 0.02 M potassium buffer (pH 7.0) was added to the enzyme solution. The reaction mixture was incubated at 80°C water bath for 10 minutes. To terminate the isomerization reaction, 60 μl of reaction mixture was transferred to a tube containing 540 μl 0.1 M HCl solution. D- fructose formation at the end of the isomerization reaction was determined by cysteine-carbazole-sulfuric acid method described by Dische and Borenfreund (1951). Following the termination of enzymatic reaction, 1.8 ml of 70% (v/v) sulfuric acid

was added to the tube. Afterwards, 60 µl of freshly prepared 1.5% (w/v) cysteine-HCl, 60 µl of freshly prepared 0.12% (w/v) carbazole in 95% ethanol was added to the tube, respectively. The mixture was vortexed and incubated at room temperature for 30 minutes. D-fructose concentration was determined by measuring the absorbance with UV-Vis spectrophotometer at 560 nm. Calibration curve representing absorbance values with respect to D-fructose concentration is presented in Appendix B.

3.7.3 Glucose Concentration

In order to detect glucose concentration (g L^{-1}) in the fermentation medium, glucose analysis kit obtained from Biasis, Ankara was used. The analysis is based on glucose oxidation method and glucose concentrations are measured by UV-Vis spectrophotometer at 505 nm wavelength (Boyacı *et al.*, 2005).

$$D-glucose + O_2 + H_2O \xrightarrow{\text{Glucocose oxidase}} Gluconate + H_2O_2 \qquad 3.4$$

$$H_2O_2 + 4-aminoantipyrine + Phenol \xrightarrow{\text{Peroxidase}} Iminoquinone + H_2O \quad 3.5$$
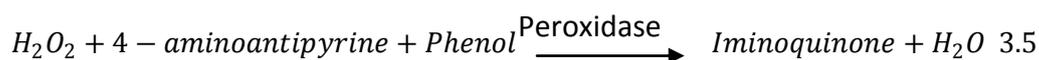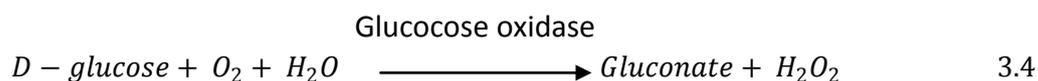
The enzyme glucose oxidase converts D-glucose and water into gluconate and peroxide in the first reaction step (equation 3.4). Peroxide, which is produced as a result of the first reaction, then reacts with 4- aminoantipyrine and phenol by peroxidase in the second reaction step (equation 3.5). At the end of the second reaction, iminoquinone compound which has a red color and equimolar with glucose is utilized to determine glucose concentration. The analysis kit, obtained from Biasis, Ankara, consists of a glucose reactive mixture containing glucose oxidase, peroxidase and 4-aminoantipyrin, a buffer solution containing phenol and potassium dihydrogen phosphate compounds.

During the analysis, samples were diluted with ultra pure water if the glucose concentrations of the sample solutions were above 2 g L^{-1}. 2 ml ultra pure water was added to tubes. Following the addition of 400 µl of buffer solution, 50 µl of sample solution was added to the tubes. Addition of glucose reactive mixture started the coloring reaction. Thereafter, tubes were incubated at 37°C water bath for 20 min and red color obtained at the end of the assay was measured using UV-Vis spectrophotometer at 505 nm wavelength. In order to convert absorbance values obtained at 505 nm to concentration values, standard glucose solutions were prepared and analyzed to obtain a calibration curve (Appendix C).

3.7.4 Organic Acid Concentration

Organic acid concentrations detected by High Performance Liquid Chromatography (HPLC) (Waters, Alliance 2695). System conditions for HPLC analysis are presented in Table 3.19. Reversed phase HPLC method was used by the Capital Optimal ODS-5Xm column (Capital HPLC, West Lothian, UK). A mobile phase consisting of 3.12% (w/v) NaH_2PO_4 and 0.62x10-3% (v/v) H_3PO_4 was used. Samples were filtered by 45 µm filters (ACRODISC CR PTFE) and diluted using the mobile phase. Filtered and diluted samples were given through the system and organic acid concentrations were determined by calculating areas under the related peaks on the chromatogram for each sample. These areas were converted to concentrations by using calibration curves (Appendix D) obtained from chromatograms of standard organic acid solutions.

Table 3.19 System conditions for HPLC to detect organic acid concentration

Column	Capital Optimal ODS, 5 µm
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Mobile phase flow rate	0.8 ml min^{-1}
Column temperature	30 ^{o}C
Detector and wavelength	Waters 2487 Dual absorbance detector, 210 nm
Injection volume	5 µl
Analysis period	15 min

CHAPTER 4

RESULTS AND DISCUSSION

In this M.Sc. study, thermostable glucose isomerase (EC 5.3.1.5) production capacities of two metabolically engineered microorganisms, *Escherichia coli* and *Pichia pastoris*, were investigated. In this context, *Pichia* cells were transfected with pPICZ-A expression vector containing GI coding gene (*xylA*) of *Thermus thermophilus* in order to construct intracellular GI producing recombinant *P. pastoris*. Following the selection of the true transformant, effects of different initial sorbitol concentrations on the cell growth and GI activity were investigated.

In the second part of the study, it was focused on feeding strategy development experiments using *E. coli* BL21 (DE3) pLysS carrying pRSETA::*xylA*$_{int}$. A laboratory scale air-filtered shake bioreactor experiment program was designed to investigate the effect of the media containing carbon sources, i. e., hydrolyzed and unhydrolyzed molasses, sucrose and glucose + fructose separately on the growth of recombinant *E. coli*. Thereafter, feeding strategy development studies were carried out to investigate the effects of pulse and continuous feeding of molasses on the GI production, cell and by-product formations, in semi-batch operation.

4.1 Recombinant Intracellular Thermostable Glucose Isomerase Production by *P. pastoris*

4.1.1 Developing Recombinant Thermostable Glucose Isomerase Producing *P. pastoris*

The research program to construct recombinant *P. pastoris* producing intracellular thermostable GI is presented schematically in Figure 4.1. pPICZ-A::*xylA*$_{int}$ plasmid was used for the transfection (Ata, 2012). The first step of constructing recombinant *P. pastoris* was isolation of plasmid from *E. coli* TOP 10 cells carrying pPICZ-A::*xylA*$_{int}$. Therefore, a single colony was picked and subjected to alkaline lysis plasmid isolation method as explained in Section 3.5.1.
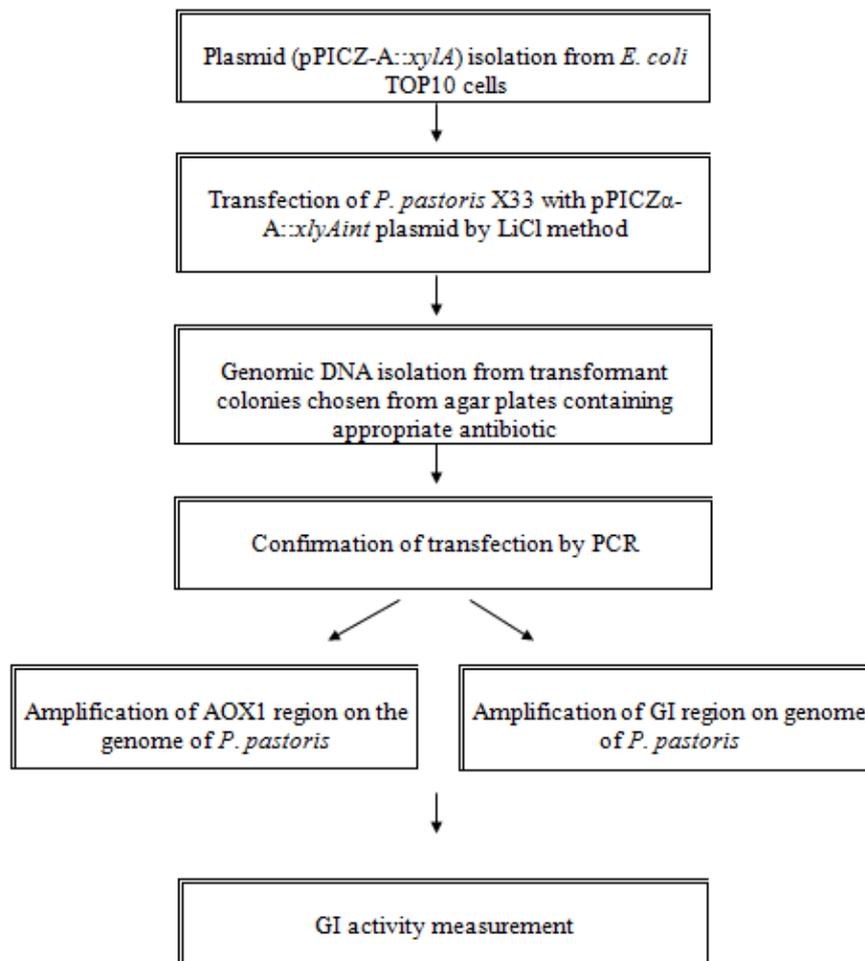
Figure 4.1 Recombinant *P. pastoris* development steps.

Isolated plasmids were loaded on an agarose gel for electrophoresis (Figure 4.2). Isolated recombinant plasmids were larger DNA molecules than the empty plasmid since they contained the gene of interest. Therefore, they ran slower in agarose gel, as expected. Isolated recombinant plasmids pPICZ-A::$xylA_{int}$ were subjected to restriction digestion as explained in Section 3.5.3 to be used for the transfection of *P. pastoris*.

4.1.1.2 Transfection of *P. pastoris* X33

Linear plasmid was obtained with *SacI* endonuclease digestion of pPICZ-A::$xylA_{int}$. Linearization was provided at the *AOX1* promoter region to enable double integration at *AOX1* region to the genome of *P. pastoris*. After purification of linear plasmid, transfection of *P. pastoris* was performed with LiCl method as explained in Section 3.5.4. A schematic representation of the plasmid integration into the genome of *P. pastoris* is presented in Figure 4.3.
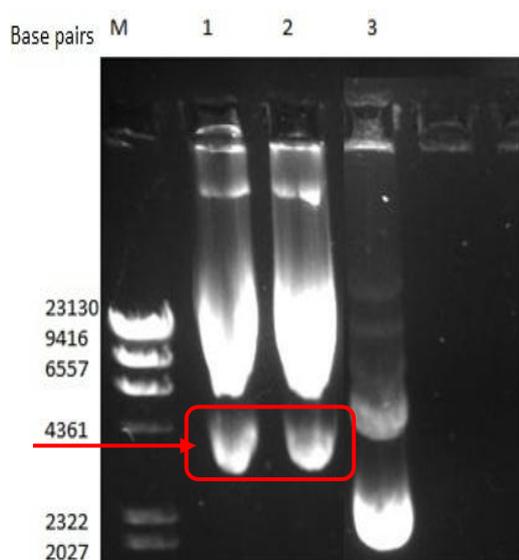
Figure 4.2 Agarose gel electrophoresis image of plasmid isolation.M: Marker (λ DNA/HindIII), Lane 1-2:Isolated pPICZ-A::$xylA_{int}$ plasmids from *E. coli* TOP10 (circular form), Lane 3: Empty pPICZ-A (circular form).
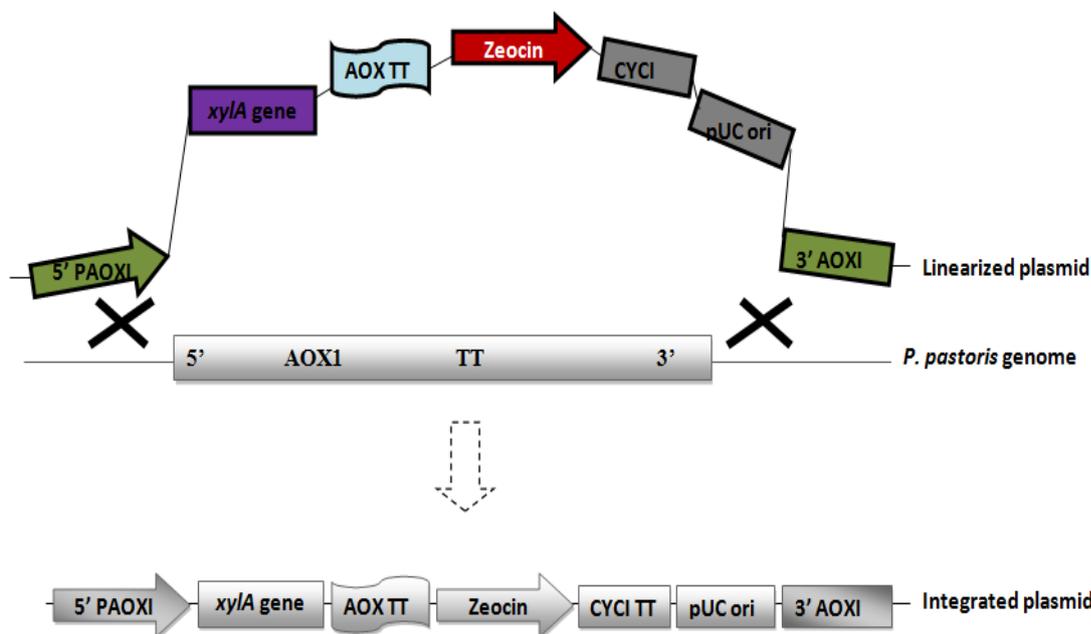
Figure 4.3 Schematic representation of pPICZ::*xylA*~int~ integration into the genome of *P. pastoris*.

4.1.2 Microorganism Selection

After transfection of *P. pastoris*, colonies were selected from agar plates and used for genomic DNA isolation. Since the linearized plasmid was integrated into the genome of *P. pastoris*, isolated genomic DNA from selected colonies was used as templates in the PCR for validation of transfection.

4.1.2.1 Microorganism Selection via PCR

PCR was used to validate the accuracy of the transfection. Out of 20 colonies grown on agar plates, one showed positive results in the amplification of both *AOX1* and *xylA* genes using *AOX1* and *xylA* specific primers presented in Table 3.14. The recombinant colony was named as *P. pastoris* S18 and used for further experiments. The agarose gel electrophoresis images of PCR results are presented in Figure 4.4 and 4.5.
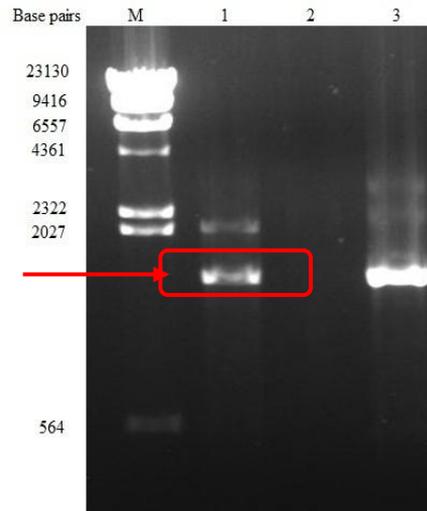
Figure 4.4 Agarose gel electrophoresis image of PCR results perfomed by using genomic DNA isolated from S18 as template for amplification of *AOX1* region. M: Marker (λ DNA/HindIII), Lane 1: *P. pastoris* S18 *AOX1* region (1484 bp), Lane 2: Negative control, Lane 3: Positive control, *P. pastoris AOX1* region.
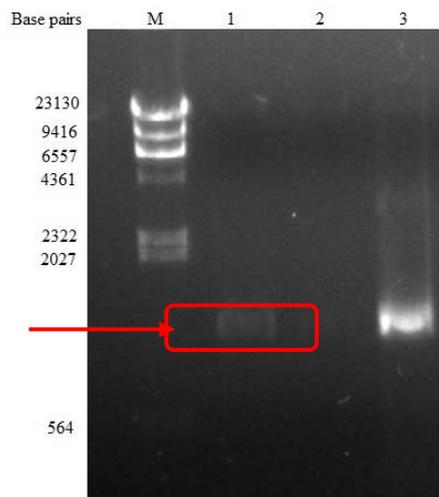
Figure 4.5 Agarose gel electrophoresis image of PCR results perfomed by using genomic DNA isolated from S18 as template for amplification of *xylA* gene. M: Marker (λ DNA/HindIII), Lane 1: *xylA* gene (1164 bp) present in *P.pastoris* S18 genome, Lane 2: Negative control, Lane 3: Positive control, *xylA* gene.

After the selection of the true transformant, a laboratory scale air-filtered shake bioreactor experiment was performed using the medium containing only methanol as the carbon source to determine the glucose isomerase production capacity of *P. pastoris* carrying pPICZ-A*::xylA*$_{int}$. At t=24h in the production phase, GI activity was reported as A=545 UL^{-1} using methanol as sole carbon source.

4.1.2 Effects of Sorbitol Concentration on Cell Growth and Thermostable GI Production of *P. pastoris* S18

The effects of initial sorbitol concentration (C_{S0}=10 g L^{-1}, C_{S0}=30 g L^{-1} and C_{S0}=50 g L^{-1}) on the glucose isomerase activity and cell growth of *P. pastoris* were investigated in laboratory scale air-filtered shake bioreactors containing production media presented in Table 3.7.

The effects of sorbitol concentration on the cell growth are presented in Figure 4.6. The results revealed that the cell concentration profiles showed an increasing trend and reached to a maximum at C_{S0}=30 g L^{-1}. However, further increase to C_{S0}=50 g L^{-1} caused a decrase in the cell concentration. The highest cell concentration was obtained as C_X=4.2 g L^{-1} with C_{S0}=30 g L^{-1} at t=36h; whereas, C_{S0}=10 g L^{-1} and C_{S0}=50 g L^{-1} resulted C_X=4.0 g L^{-1} and C_X=3.7 g L^{-1} cell concentrations at t=36h of the bioprocess, respectively. When with and without co-carbon source sorbitol results are compared; 1.27-fold incerase in the cell concentration was obtained with the use of C_{S0}=30 g L^{-1} sorbitol.

The highest GI activity was obtained at C_{S0}= 30 g L^{-1} at t= 24h as A= 792.9 U L^{-1} in consistent with the cell growth. When with and without co-carbon source sorbitol results are compared, 1.45-fold incerase in GI activity was obtained with the use of C_{S0}= 30 g L^{-1} sorbitol. C_{S0}= 10 g L^{-1} and C_{S0}= 50 g L^{-1} yielded A= 537.7 U L^{-1} and A= 439.5 U L^{-1} GI activity at t= 24h of the bioprocess, respectively (Figure 4.7). Sorbitol concentration up to $C_{S0=}$ 30 g L^{-1} favored both the cell growth and GI activity. However, when it was increased to 50 g L^{-1}, GI activity was affected in a negative manner as in the cell growth. Therefore, it is concluded that C_{S0}=30 g L^{-1}

sorbitol concentration is optimal for the cell growth and protein production. This laboratory scale bioreactor experiment also revealed the promoting effect of co-substrate sorbitol on cell growth and protein expression, as reported by Jungo *et al.* (2007) and Çelik *et al.* (2009).
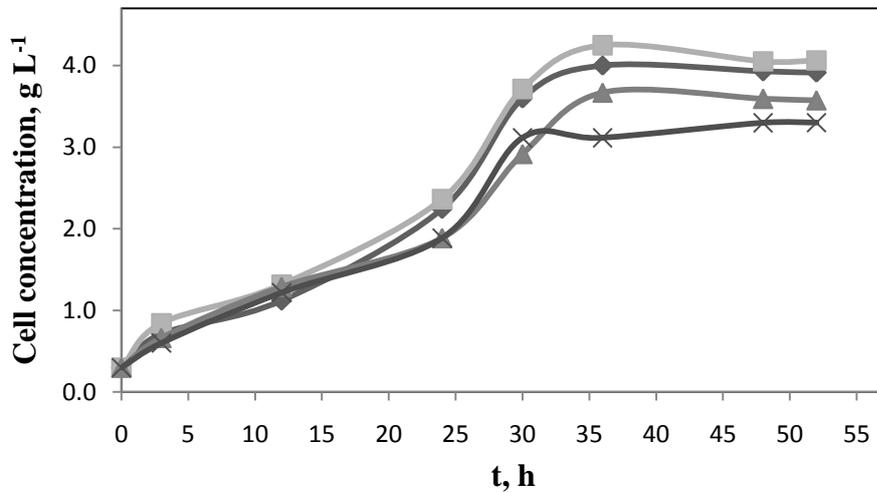
Figure 4.6 Effects of sorbitol concentration on cell growth *P. pastoris*: C_{S0}=10 g L^{-1}(♦),C_{S0}=30 g L^{-1}(■),C_{S0}=50 (▲), no-sorbitol (x).
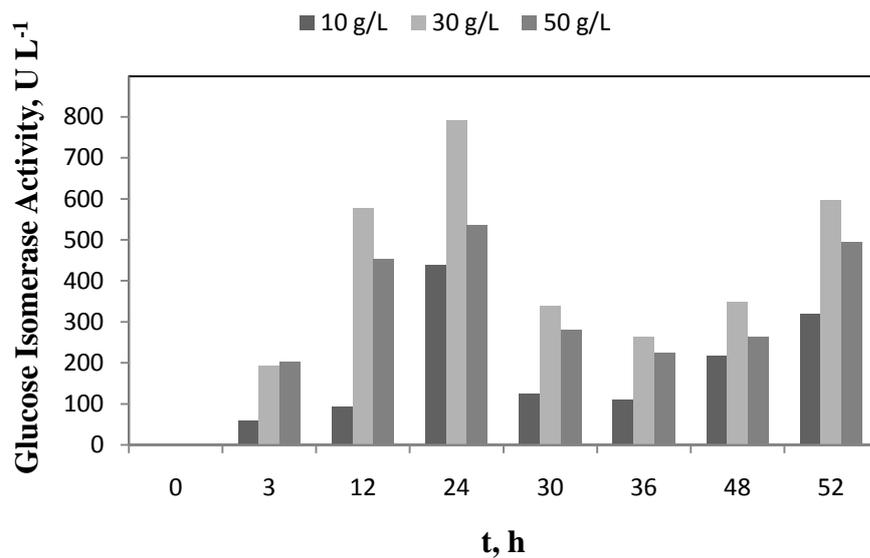
Figure 4.7 Effects of sorbitol concentration on GI activity of *P. pastoris*.

In the M.Sc. study of Ata (2012), the effects of sorbitol concentration (C_{S0}=10 g L^{-1}, 30 g L^{-1} and 50 g L^{-1}) on the extracellular GI production were investigated in laboratory scale shake bioreactors using *P. pastoris* carrying *xylA* gene of *Thermus thermopilus* in pPICZα-A plasmid. The highest GI activity was obtained with C_{S0}=50 g L^{-1} as A=3.6 U L^{-1}. In intracellular GI production, 151.4-fold increase in GI activity was achieved in the designed air-filtered shake bioreactor experiment. Eukaryotic folding and subsequent secretion process of a protein depend on many factors and components. Increased foreign gene expression rate within the cell could limit the rate of another step. Then, this step becomes a barrier in the secretion pathway which principally hinders protein exit from endoplasmic reticulum (Shuster, 1991). Hence, the main problem in lower extracellular production could be because of the problems occurring in secretion process. In addition, it is known that recombinant protein secretion from yeast depends on the foreign protein that is going to be expressed, signal sequence and host strain (Hashimoto *et al.*, 1998). Therefore, another reason of differences in GI activity can be because of the inefficiency of signal sequence (α-factor of pPICZ-A).

In the Ph.D. study of Angardi (2011), GI gene obtained from *Pyrococcus furiosus* was expressed in *P. pastoris* both intracellularly and extracellularly. In air filtered shake bioreactor experiments using BMMY medium A=209 U L^{-1} and A=342 U L^{-1} GI activities were obtained for extracellular and intracellular production, respectively. In this study, intracellular GI expression of *Thermus thermophilus* without addition of sorbitol yielded 1.6-fold increase in the activity when compared to Angardi (2011). The difference in the activity is probably related with the origin of the gene of interest.

4.2 Recombinant Intracellular Thermostable Glucose Isomerase Production by *E. coli*

4.2.1 Effects of Different Carbon Sources on Cell Growth of *E .coli*

Laboratory scale shake bioreactor experiments were carried out to investigate whether *E. coli* could utilize sucrose as a carbon source since some contradictions about sucrose utilization by *E. coli* existed in the literature. Therefore, the effects of carbon sources (Table 3.10) were investigated for the growth of *E. coli* (Figure 4.8). The highest cell concentration was obtained in the medium containing C_{M0}=30 g L^{-1} hydrolyzed molasses as C_X = 1.65 g L^{-1}. When glucose+ fructose was used as the carbon sources together at C_{G0} = 8 g L^{-1} and C_{F0}= 8 g L^{-1}, cell concentration was obtained as C_X = 1.45 g L^{-1}. Thus, it was concluded that components in molasses supported the *E. coli* growth and resulted in higher cell concentration. Unhydrolyzed molasses-based and sucrose-based medium resulted in C_X = 0.55 g L^{-1} and C_X = 0.24 g L^{-1}, the cell concentration, respectively. Concentration of the cells at the beginning of the production process was C_{X0}=0.20 g L^{-1} meaning that no significant increase in the cell concentration of *E. coli* BL21 (DE3) pLysS was observed when defined sucrose-based medium was used.

Based on the data obtained from the experiments, it was concluded that since unhydrolyzed molasses contained sucrose as the main carbon source and no significant cell growth was observed in the sucrose-based medium, higher cell concentration obtained with the untreated molasses was due to the positive effects of the components in molasses on cell growth rather than sucrose utilization of *E. coli* BL21 (DE3) pLysS.

Ye *et al.* (2010) reported that untreated cane molasses could be used for the production of heterologous proteins by *E. coli* Rosetta (DE3) pLysS. They conducted an experiment which was carried out using LB medium with sucrose at the concentration of 10 g L^{-1} and cells reached to the concentration of OD$_{600}$=11.5. Using the data, they mentioned about the possibility of a sucrose hydrolase gene in the genome of *E. coli* Rosetta (DE3) pLysS. The most probable reason that cell

proliferation occurred in sucrose containing LB medium was owing to utilization of components of yeast extract presented in LB as carbon source.

Considering its positive effect on the cell growth of *E. coli* BL21 (DE3) pLysS the hydrolyzed molasses-based medium was chosen for bioreactor feeding strategy development experiments.

In a laboratory scale air-filtered shake bioreactor experiment program with hydrolyzed molasses-based medium, using *E. coli* BL21 (DE3) pLysS carrying pPRSETA::*xylA*$_{int}$, GI activity was A=2312 U L^{-1} which was 3.17 fold higher than that of *P. pastoris*. The difference in the GI activity is probably related with the origin of the gene of interest, as *xylA* is taken from a bacterium and expressed in yeast, amino acid codons might not be appropriate. Thus, for GI production by *P. pastoris* codon optimization can be applied to increase the production levels. However, in this study, based on the GI production levels of *P. pastoris* and *E. coli*, *E. coli* was preferred as a potential GI producer for bioreactor feeding strategy develeopment experiments.
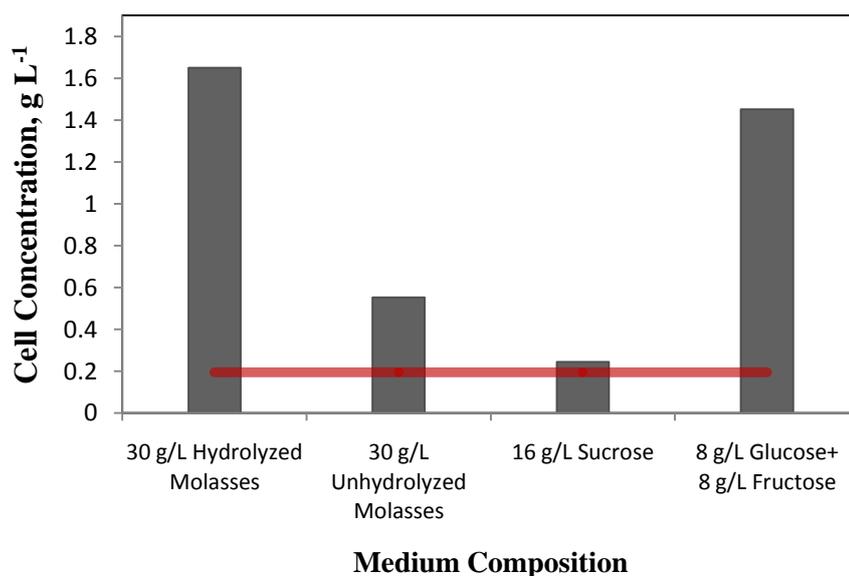
Figure 4.8 Effects of carbon sources on cell growth of *E. coli* at=12h (Line represents the cell concentration at t=0h: 0.20 g L^{-1}).

4.2.2 Development of Feeding Strategies for Production of GI in Pilot Scale Bioreactor

In this M.Sc. study, four sets of bioreactor experiments were performed using the medium presented in Table 3.11. The experiments were started batch-wise and continued with different feeding strategies in semi-batch operation. The batch phase of all bioreactor experiments were carried out at the same inital molasses concentration of C_{M0}=30 g L^{-1}. Considering the inhibitory effects of the glucose concentration above 2 g L^{-1} on aerobic growth of the cell and expression of recombinant gene product (Vila and Villaverde, 1993), the batch phase was continued until glucose concentration decreased ca. 2 g L^{-1} in all strategies. When the glucose concentration reached to non-inhibitory levels in the bioreactor, which corresponded to t=7h of the bioprocess, effects of the continuous or pulse feeding of the hydrolyzed molasses were investigated. In the semi-batch operation, hydrolyzed molasses was fed to the system according to equation 3.1 where glucose and fructose were dual carbon sources.

Strategies used in the bioreactor experiments are explained in Table 4.1. The first semi-batch bioreactor experiment, M-0.05 was designed to investigate the effect of the nitrogen and phosphorus source in semi-batch operation with pre-determined specific growth rate of μ_0=0.05 h^{-1}. After the batch phase conducted until t=7h of the bioprocess, the molasses feed containing $(NH_4)_2HPO_4$ with the same ratio as in the initial medium was supplied to the bioreactor continuously.

The second strategy M-0.1 was designed to investigate the effect of the pre-determined specific growth rate of μ_0=0.1 h^{-1}. After the batch phase, continuous molasses feeding together with $(NH_4)_2HPO_4$ was started in the semi-batch operation.

The third strategy, PM-0.05 was designed using a bioreactor pulse feeding after the batch phase. Glucose concentration in the bioreactor was increased to its initial value with pulse feeding of molasses at t=7h. Following the pulse feeding, glucose concentration decreased to non-inhibitory levels at t=10h of the bioprocess. Hence, continuous feeding of molasses containing $(NH_4)_2HPO_4$ with a pre-determined specific growth rate of μ_0=0.05 h^{-1} was started at t=10h.

In the fourth strategy 2PM-0.05, compared to PM-0.05, second molasses pulse feeding was made at t=10h to increase glucose concentration to its initial value. Following the second pulse, continuous feeding of molasses containing $(NH_4)_2HPO_4$ based on pre-determined specific growth rate $\mu_0=0.05$ h^{-1} was started after the depletion of glucose to the desired concentration which corresponded t=11h of the bioprocess.

The variations of pre-calculated continuous volumetric feed rate Q(t) functions based on the specific growth rates (μ_0) using equation 3.1 are graphically presented in Figure 4.9.
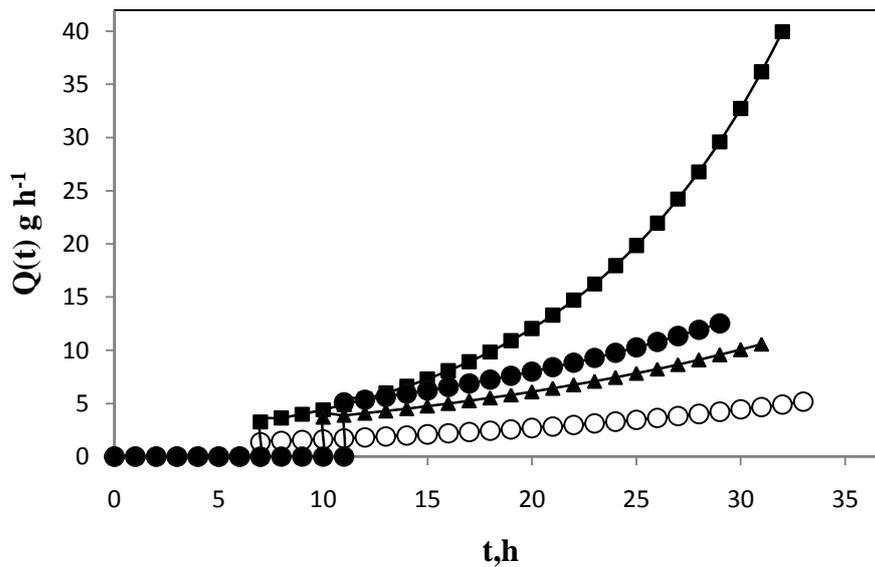
Figure 4.9 Pre-determined feeding profiles with the cultivation time and feeding strategy applied:M-0.05: Q(t)=1.35exp(0.05t) (○), M-0.1: Q(t)=3.28 exp(0.1t) (■), PM-0.05: Q(t)=3.70exp(0.05t) (▲), 2PM-0.05: Q(t)=5.1 exp(0.05t) (●).

Table 4.1 Strategies applied in bioreactor experiments

Abbreviation of the Strategy	Explanation of the Strategy Applied
M-0.05	♦Batch phase started with C_{M0}=30 g L^{-1} (C_{G0}=8 g L^{-1}, C_{F0}=8 g L^{-1}) and conducted between t=0-7h. ♦Semi-batch phase conducted between t=7-34h. Exponential feeding rate of molasses containing $(NH_4)_2HPO_4$ was calculated based on μ_0=0.05 h^{-1}.
M-0.1	♦Batch phase started with C_{M0}=30 g L^{-1} (C_{G0}=8 g L^{-1}, C_{F0}=8 g L^{-1}) and conducted between t=0-7h. ♦Semi-batch phase conducted between t=7-33h. Exponential feeding rate of molasses containing $(NH_4)_2HPO_4$ was calculated based on μ_0=0.1 h^{-1}.
PM-0.05	♦Batch phase started with C_{M0}=30 g L^{-1} (C_{G0}=8 g L^{-1}, C_{F0}=8 g L^{-1}) and conducted between t=0-7h. ♦Pulse feeding of molasses containing $(NH_4)_2HPO_4$ was made at t=7h to increase C_G to its initial value 8 g L^{-1}. ♦Semi-batch phase conducted between t=10-32h. Exponential feeding rate of molasses containing $(NH_4)_2HPO_4$ was calculated based on μ_0=0.05 h^{-1}.
2PM-0.05	♦Batch phase started with C_{M0}=30 g L^{-1} (C_{G0}=8 g L^{-1}, C_{F0}=8 g L^{-1}) and conducted between t=0-7h. ♦The first pulse feeding of molasses containing $(NH_4)_2HPO_4$ was made at t=7h to increase C_G to its initial value 8 g L^{-1}. ♦The second pulse feeding of molasses containing $(NH_4)_2HPO_4$ was made at t=10h to increase C_G to its initial value 8 g L^{-1} again. ♦Semi-batch phase conducted between t=11-30h. Exponential feeding rate of molasses containing $(NH_4)_2HPO_4$ was calculated based on μ_0=0.05 h^{-1}.

4.2.2.1 Substrate Consumption

All bioreactor experiments were conducted using hydrolyzed molasses-based medium. Since, glucose and fructose are the main carbon sources in the hydrolyzed molasses-based medium, variations in glucose and fructose concentrations with the cultivation time and feeding strategy applied are presented in Figure 4.10 and 4.11, respectively. Batch phase in all bioreactor experiments were conducted at almost the same initial cell concentration, therefore substrate consumption profiles were almost the same in all bioreactor experiments, as expected.

Initial fermentation medium containing C_{M0}=30 g L^{-1} molasses contains C_{G0}=8 g L^{-1} glucose and C_{F0}=8 g L^{-1} fructose due to the pretreatment of molasses. After t=4h of the bioprocess a significant decrease in the glucose concentration was observed in all the bioreactor experiments. At t=7h of the bioprocess, glucose reached to non-inhibitory levels. Therefore, pulse and continuous feeding in semi-batch operation was started at t=7h since lower glucose concentrations within the fermentation medium favored expression of the recombinant gene, cell growth (Shiloach and Fass, 2005) and prevented acetate formation (Babaeipour *et al.*, 2008). In all strategies, no significant accumulation of glucose was observed during the continuous feeding of molasses.

In Figure 4.11, relatively slight depletion of fructose was observed with the cultivation time. Hence, it can be inferred that in the presence of both glucose and fructose in the fermentation medium, the preference of the cells was towards glucose. In all bioreactor experiments, feeding strategies were developed according to the concentration of glucose in the fermentation medium. In the first bioreactor experiment M-0.05, no significant fructose accumulation was observed. However, when the pre-determined specific growth rate was increased to 0.1 h^{-1}, significant fructose accumulation was observed after t=24h and fructose concentration increased to C_F=8.5 g L^{-1} by the end of the process. Similarly, in the strategy 2PM-0.05, accumulation of fructose was observed following the continuous molasses feeding. After the introduction of second pulse to the system, fructose concentration decreased from C_F=13.1 g L^{-1} to C_F=5.3 g L^{-1}, remained constant at a relatively high

value between t=14-28h and started to accumulate after t=28h. By the end of the process, fructose concentration reached to $C_F=12.3$ g L^{-1}. The possible reason was probably high rate of feed due to increased cell concentration owing to second pulse. In the literature, it was stated that fructose reduced *lac* expression similar to glucose by decreasing cAMP level (Minhas, *et al.*, 2006). Therefore, these two strategies, M-0.1 and 2PM-0.05, are not favorable due to the substrate accumulation occurred towards the end of the experiments.
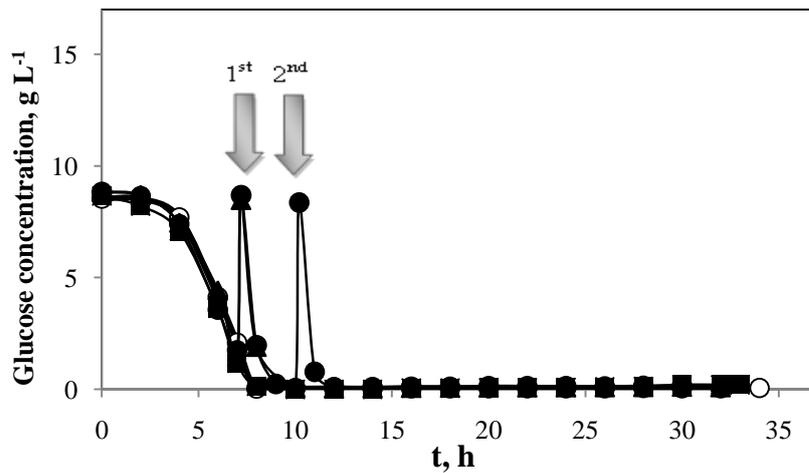
Figure 4.10 Variations in glucose concentrations with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●). (Arrows indicate the molasses pulses).
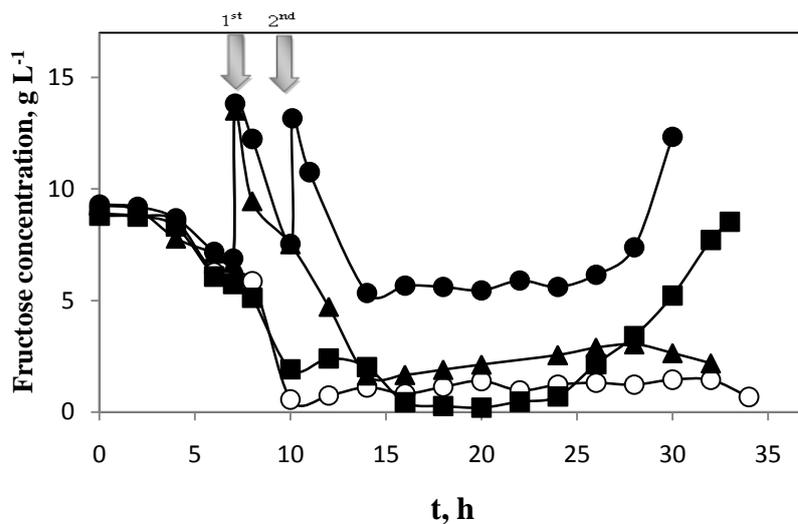
Figure 4.11 Variations in fructose concentrations with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●). (Arrows indicate the molasses pulses).

4.2.2.2 Effects of Feeding Strategies on the Cell Growth

Variations in cell concentrations with the cultivation time and feeding strategy applied are presented in Figure 4.12. Cell concentration profiles showed a similar trend until the pulse feeding or continuous feeding was started. Among all semi-batch exponential feeding experiments, the highest cell concentration was obtained with M-0.1 as C_X=17.9 g L^{-1} at t=28h as a result of high exponential feed rate. When M-0.05 and PM-0.05; PM-0.05 and 2PM-0.05 are compared, the significant effect of pulses on the cell growth can be observed from the cell concentration profiles. The maximum cell concentration obtained at M-0.05 was C_X=7 g L^{-1}. Molasses pulse feeding increased the maximum cell concentration to C_X= 13.3 g L^{-1} in PM-0.05 which was 1.9-fold of M-0.05. In parallel, 2PM-0.05, the maximum cell concentration increased to C_X= 16.5 gL^{-1} which was 1.24-fold of PM-0.05.
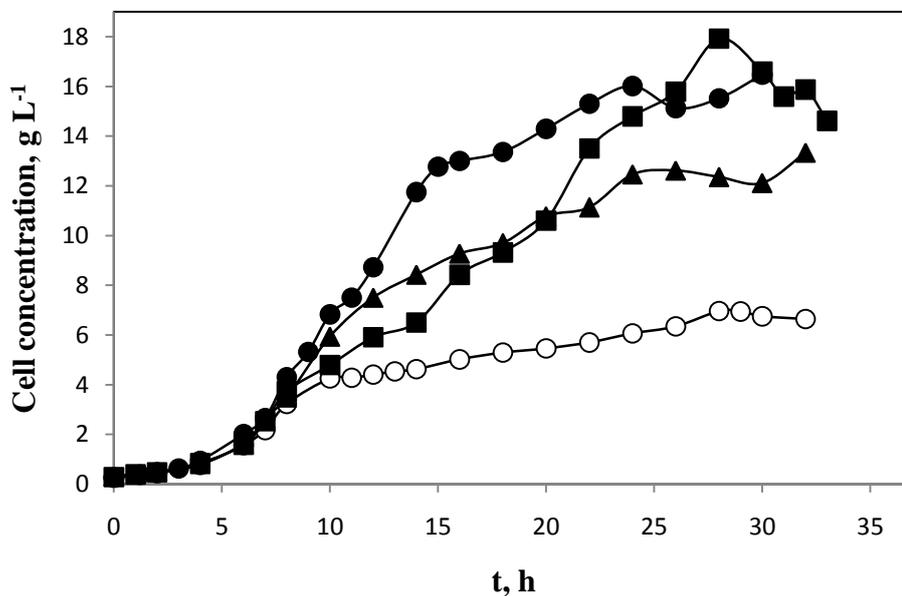
Figure 4.12 Variations in cell concentrations with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●).

Pulse feeding also affected the beginning time of stationary phase. Since pulse feeding contained molasses, nitrogen and phosphorus source, it fulfilled the nutritional requirement of the cells. Therefore, exponential growth phase lasted longer. Similarly, when M-0.05 and M-0.1 are compared, higher feed rate caused higher cell concentrations in M-0.1 by providing more nutrients for consumption of the cells.

In literature, Çalık and Levent (2000-b) obtained the highest cell concentration as 8.04 g L^{-1} (t=24h) using molasses-based medium with a developed pulse feeding strategy for BAL production by *E. coli* BL21 (DE3) pLysS. On the other hand, Angardi and Çalık (2013) used the same strain of *E. coli* carrying pRSETA::$xylA_{int}$ which was also used in this study, and obtained the highest cell concentration as C_X= 9.6 g L^{-1} by molasses feeding in semi-batch operation with a pre-determined specific rate of μ_0=0.15 h^{-1}. In the M.Sc. study of Akdağ (2013), the highest cell concentration of *E. coli* W was obtained as C_X=18.4 g L^{-1} (t=26h) with exponential feeding μ_0=0.05 h^{-1} after two molasses pulses in semi-batch operation mode.

4.2.2.3 Effects of Feeding Strategies on the Enzyme Activity

Variations in volumetric GI activities with the cultivation time and feeding strategy applied are presented in Figure 4.13. GI activity profiles of all the semi-batch bioreactor experiments with different strategies showed an increasing trend after molasses feeding was started in semi-batch operation. However, the cultivation time for maximum GI activity varied under the influence of strategy applied.

In M-0.05 the maximum volumetric activity was obtained as A=18137 U L^{-1} at t=32h. Angardi and Çalık obtained A=16399 U L^{-1} GI activity using *E. coli* BL21 (DE3) pLysS carrying pRSETA::$xylA_{int}$ by feeding molasses at the same specific rate of μ_0=0.05 h^{-1}. Therefore, as a result of addition of $(NH_4)_2HPO_4$ as nitrogen and phosphorus source, 1.1-fold increase was obtained in the GI activity. Considering the positive effect of $(NH_4)_2HPO_4$ in feed, semi-batch process with $(NH_4)_2HPO_4$ in the continuous feed was used in all bioreactor experiments.

In M-0.1, the effect of pre-determined specific growth rate was investigated. Even though the maximum cell concentration was attained with this strategy, GI activity reached up to $A=9990.2$ U L^{-1} at t=22 h. It can be interpreted that, high feed rate favored cell proliferation instead of enzyme production in this strategy. Hence, in the rest of the strategies pre-determined specific growth rate of $\mu_0=0.05$ h^{-1} was applied in continuous feeding.

The affect of one pulse containing $(NH_4)_2HPO_4$ was investigated in PM-0.05 strategy. 1.6-fold increase in GI activity was obtained in PM-0.05 strategy after the addition of molasses pulse to the system. Moreover, with pulse feeding, cultivation time where the maximum GI was obtained shifted to an earlier time when compared to M-0.05.

Afterwards, the effect of the second pulse was examined in the strategy 2PM-0.05. As stated before, after the second pulse of 2PM-0.05, fructose was started to accumulate within the fermentation medium in this strategy. Therefore, due to substrate accumulation, the highest enzyme was obtained as $A=14248.1$ UL^{-1} which corresponded about 2 fold- decrease in GI activity compared to the previous strategy.

Among the bioreactor experiments, the highest GI activity was obtained as $A=29050$ UL^{-1} using one molasses pulse condition, PM-0.05 at t=26h. With the developed strategy thermostable GI was increased 1.77-fold when compared to Angardi and Çalık (2013).

In the M.Sc. study of Akdağ (2013), untreated molasses-based continuous feeding strategy in semi-batch operation mode was developed for GI production using a different strain, sucrose utilizing *E.coli* W. Effect of one pulse and two pulses without $(NH_4)_2HPO_4$ on GI activity was investigated and significant increase was obtained. Addition of one pulse increased the activity about 1.2-fold and two pulses about 1.77-fold compared to no-pulse condition. Moreover, when two pulses strategy contained $(NH_4)_2HPO_4$ as nitrogen source, the highest GI activity was obtained with a 1.25-fold increase. However, in this study, the highest GI activity was obtained with one pulse strategy instead of two pulse strategy, which was probably due to the accumulation of fructose.
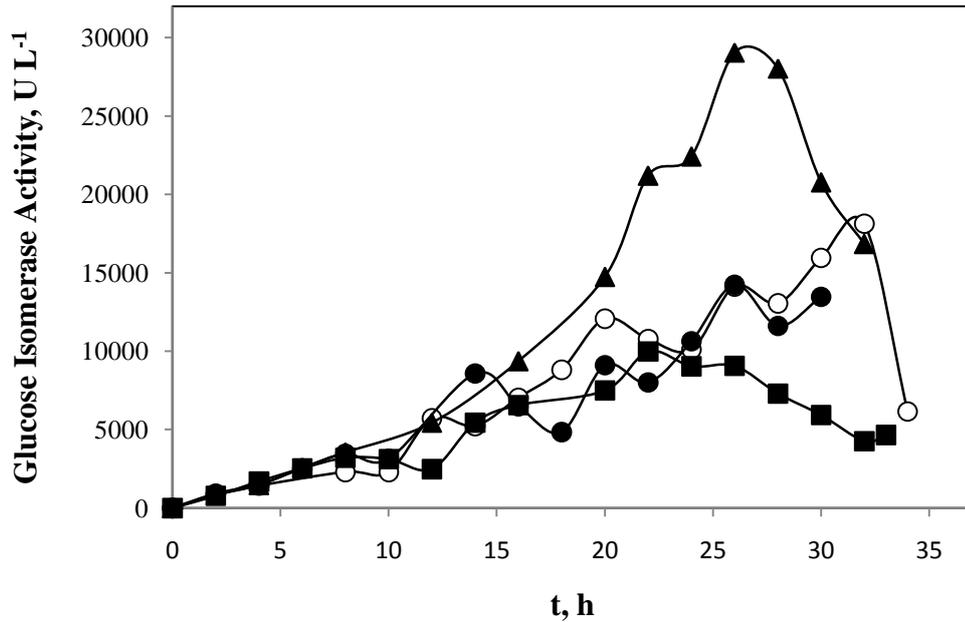
Figure 4.**13** Variations in GI activities with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●).

Specific GI activity was calculated as GI activity per gram of cell. Variations in specific activity with the cultivation time for the feeding strategies applied are presented in Figure 4.14. Specific glucose isomerase acitivy showed an increasing profile at the beginning of the bioprocess in all feeding strategies. Then, a sharp decrease was observed for M-0.05 and 2PM-0.05 at t=2h, for M-0.1 and PM-0.05 at t=4h. However, specific GI activities of M-0.05 and PM-0.05 started to increase at t=12-14h whereas they showed a stationary profile for M-0.1 and 2PM-0.05 probably due to reaching higher cell concentrations in M-0.1 and 2PM-0.05 strategies. The highest specific GI activity was obtained as A_X=2177 U g DCW^{-1} in M-0.05 at t=32h. In terms of A_X, M-0.05 strategy is more favorable than PM-0.05 strategy where the highest volumetric activity was obtained.
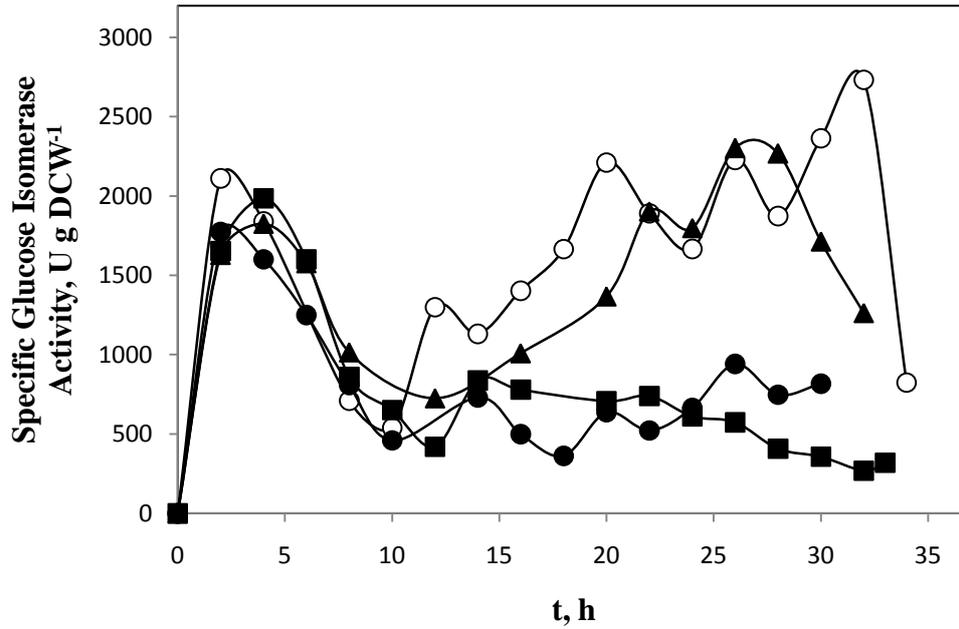
Figure 4.**14** Variations in specific GI activities with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●).

Table 4.2 Semi-batch operation mode characteristics for different strategies

Strategy	Batch operation cultivation time prior to semi-batch operation (t), h	Maximum volumetric GI			Overall values within t=0 – t=t_{max}
		Cultivation time (t_{max}), h	Glucose Isomerase Activity, U L^{-1}	Cell concentration, g L^{-1}	Total substrate consumed, g
M-0.05	7	32 (26)	18137 (14132.3)	6.63 (6.34)	34 (24)
M-0.1	7	22	9990.2	13.50	42.2
PM-0.05	10	26	29050	12.61	48.8
2PM-0.05	11	26	14248.1	15.12	64.6

4.2.2.4 Organic Acid Concentration Profiles for Feeding Strategies

Organic acid profile can be used to interpret the intracellular metabolic reactions. Acetic, formic, lactic, malic, succinic and citric acid are the organic acids excreted to the fermentation medium. Variations in organic acid and acetic acid concentrations with the cultivation time and feeding strategy applied are presented in Figure 4.15 and Figure 4.16, respectively.

It has been known that under anaerobic conditions *E. coli* produces a mixture of organic acids (Stokes, 1948). It should be noted that, during the bioreactor experiments, dissolved oxygen levels could not be maintained at 40%, especially for the strategies contained molasses pulse(s), PM-0.05 and 2PM-0.05. An increasing trend was observed for PM-0.05 and M-0.05 strategies for total organic acid production. For 2PM-0.05 and M-0.1, total organic acid concentrations were decreased sligthly by the end of the bioprocess. *E. coli* utilizes organic acids as carbon sources when the carbon source becomes limited within the fermentation medium. Therefore, decreases and increases observed in organic acid profiles are the results of their uptake and formation by the cells. Lower total organic acid concentration profile observed in M-0.1 and 2PM-0.05 compared to M.0.05 and PM-0.05 inspite of higher feeding rate of molasses observed in these two strategies. It can be inferred that substrates were utilized more efficiently in terms of cell concentration and less by-products are produced in M-0.1 and PM-0.05.

The maximum acetic acid concentrations were detected as 4.76, 1.34, 4.24 and 1.77 g L^{-1} for M-0.05, M-0.1, PM-0.05 and 2PM-0.05 strategies, respectively. It was reported that acetic acid concentration (above 5 g L^{-1}) causes reduction in recombinant protein production, cell growth and reachable cell density (Eiteman and Altman, 2006; De Mey *et al.*, 2007). Therefore, it can be concluded that acetic acid concentrations fluctuated at tolerable limits for different feeding strategies.

With the best strategy in terms of GI activity PM-0.05, organic and acetic acid concentration showed an increasing profile during exponential feeding in semi-batch operation. High acetic acid concentration was detected especially after the time when the maximum GI activity was attained, which could be the reason of decreasing

activity profile starting at that time (t=28h). The highest acetic acid and organic acid concentration obtained with the best strategy PM-0.05 were detected as 4.24 g L^{-1}, (t=28h) and 5.05 (t=32) g L^{-1}, respectively.

. Shiloach *et al*. (1996) reported 2 g L^{-1} and 1 g L^{-1} acetic acid production for batch and semi-batch fermentation of *E. coli* BL21, respectively. Nevertheless, fermentation process was carried out using glucose-based medium. For different strategies applied by Angardi and Çalık (2013) using *E. coli* BL21 (DE3) pLysS carrying pRSETA::*xylA*int and molasses-based medium, the maximum acetic acid concentrations were reported as 1.72, 12.32, 18.14 g L^{-1}. Thus, it can be stated that relatively low acetic acid production was attained with the strategies applied in this study compared to Angardi and Çalık (2013). As long as nitrogen source limited conditions cause increase in acetate formation (Marzan and Shimuzu, 2011), the possible reason of this result is prevention of nitrogen limited conditions using $(NH_4)_2HPO_4$ in semi-batch feed.
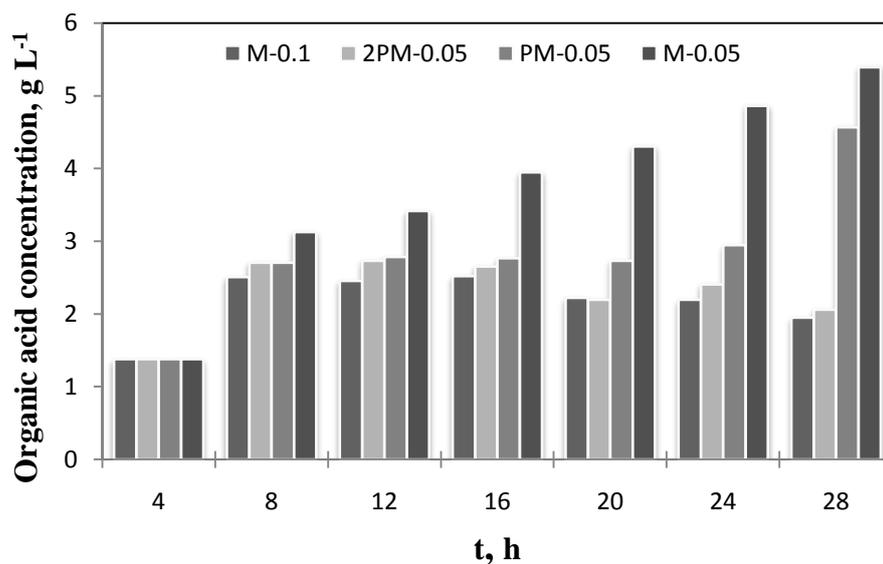
Figure 4.15 Variations in total organic acid concentrations with the cultivation time and feeding strategy applied
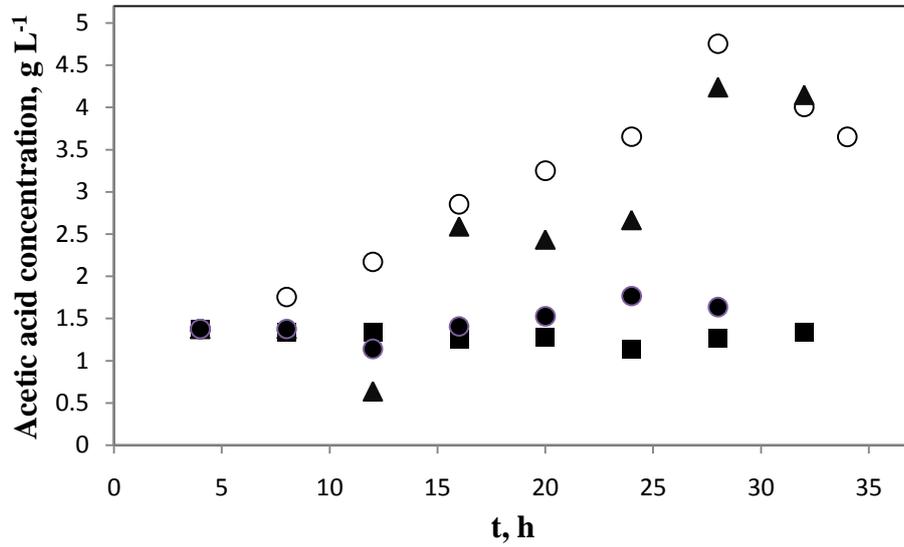
Figure 4.16 Variations in acetic acid concentrations with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●).

Table 4.3 Organic acid concentration profiles for feeding strategies

| t, h | Concentration, g L^{-1} | | | | | | |
	Acetic acid	Formic acid	Lactic acid	Malic acid	Succinic acid	Citric acid	Total
				M-0.05			
4	1.378	0.000	0.000	0.000	0.000	0.000	1.378
8	1.755	0.000	0.864	0.510	0.000	0.000	3.129
12	2.172	0.000	0.807	0.436	0.000	0.000	3.415
16	2.854	0.000	0.000	0.370	0.723	0.000	3.947
20	3.252	0.000	0.000	0.385	0.668	0.000	4.305
24	3.655	0.000	0.000	0.488	0.719	0.000	4.862
28	4.756	0.000	0.000	0.487	0.152	0.000	5.395
32	4.005	0.000	0.000	0.720	0.000	0.000	4.725
34	3.652	0.000	0.000	0.531	0.000	0.000	4.138

M-0.1 (Table 4.3 continued)

8	1.335	0.000	0.972	0.162	0.000	0.040	2.509
12	1.335	0.239	0.285	0.528	0.000	0.068	2.455
16	1.252	0.177	0.385	0.661	0.000	0.045	2.520
20	1.280	0.205	0.325	0.412	0.000	0.000	2.222
24	1.138	0.000	0.434	0.626	0.000	0.000	2.198
28	1.268	0.000	0.000	0.682	0.000	0.000	1.950
32	1.335	0.000	0.000	0.852	0.000	0.000	2.187

PM-0.05

8	1.378	0.000	1.026	0.240	0.000	0.061	2.705
12	0.638	0.072	1.504	0.571	0.000	0.000	2.785
16	2.591	0.000	0.000	0.175	0.000	0.000	2.766
20	2.437	0.043	0.000	0.251	0.000	0.000	2.734
24	2.668	0.000	0.000	0.277	0.000	0.000	2.945
28	4.239	0.124	0.000	0.202	0.000	0.000	4.565
32	4.151	0.016	0.000	0.468	0.000	0.000	5.045

2PM-0.05

12	1.140	0.000	2.132	0.599	0.000	0.000	2.731
16	1.141	0.266	1.050	0.195	0.000	0.000	2.652
20	1.407	0.000	0.000	0.214	0.000	0.000	2.181
24	1.527	0.000	0.000	0.208	0.000	0.000	2.408
28	1.768	0.000	0.000	0.292	0.000	0.000	2.060
30	1.636	0.000	0.000	0.903	0.000	0.000	2.539

4.2.2.5 Fermentation Characteristics

In Figure 4.17, variations in specific growth rates with cultivation time and feeding strategy applied are presented. For all different feeding strategies, μ values increased until t=6h, then began to decrease gradually with cultivation time after batch phase and reached a stationary phase. Between t=24-28h, μ values approached to zero then increased slightly by the end of the bioprocess for pulse strategies. In semi-batch feeding strategies except M-0. 1, it was aimed to keep μ at 0.05 h^{-1} and in M-0.1, 0.1 h^{-1}. From Figue 4.17 and Table 4.4, it can be said that calculated μ values was lower than the pre-determined values of 0.05 and 0.1 h^{-1} during semi-batch operation. Maintenance of μ value at 0.05 h^{-1} was more efficient in PM-0.05 strategy, where the highest volumetric GI activity was attained. The highest specific growth rates calculated for semi-batch operation modes are 0.04, 0.1, 0.5 and 0.04 h^{-1} for M-0.05, M-0.1, PM-0.05 and 2PM-0.05 strategies, respectively.

specific substrate consumption rate, q_S (g g^{-1} h^{-1}) and specific cell yield on substrate, $Y_{X/S}$ (g g^{-1}) were calculated considering sum of glucose and fructose consumption. q_S showed a decreasing profile with the cultivation time. A sharp decrease was observed in q_S values after t=4h for all bioreactor experiments. By the end of the process, a slight increase was observed. The maximum q_S value was obtained in M-0.1 as 0.44 g g^{-1} h^{-1} where the substrate was provided in a higher feeding rate. The highest q_S values obtained for M-0.05, PM-0.05 and 2PM-0.05 are 0.19, 0.39 and 0.19 g g^{-1} h^{-1}, respectively.

Evaluating overall specific cell yields on substrate $Y_{X/S}$ is also important, since they indicate the overall yield of the bioprocess. Overall $Y_{X/S}$ values are calculated as 0.20, 0.14, 0.20 and 0.21 g g^{-1} for M-0.05, M-0.1, PM-0.05 and 2PM-0.05 strategies, respectively. These results indicated that substrate utilization efficiency were close to each other for different semi-batch feeding strategies. However, the overall $Y_{X/S}$ values for semi-batch phase found to be lower than the $Y_{X/S}$ value (0.32 g g^{-1}) used for the calculation of pre-determined feeding rate.
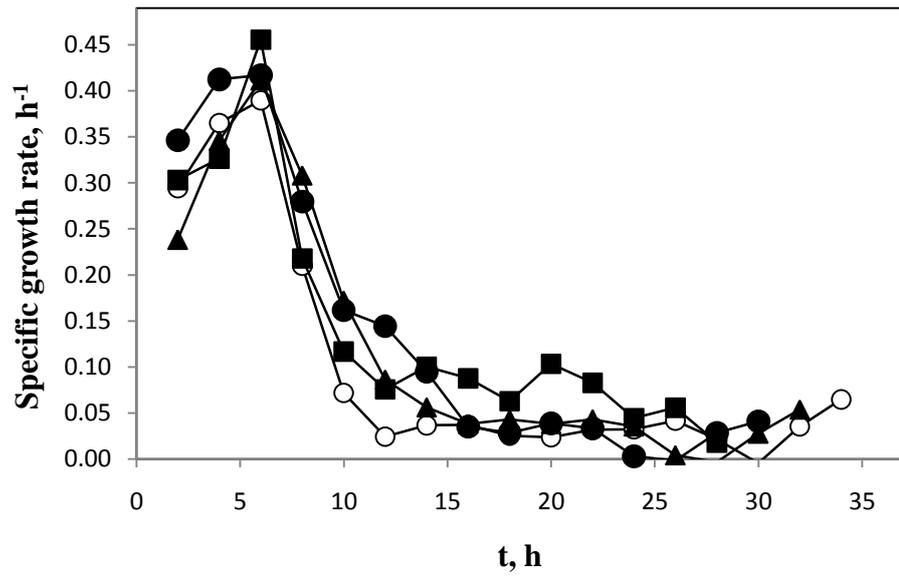
Figure 4.17 Variations in specific growth rates with the cultivation time and feeding strategy applied: M-0.05 (○), M-0.1 (■), PM-0.05 (▲), 2PM-0.05 (●).

Table 4.4 Fermentation characteristics

Feeding Strategy	t [h]	μ [h^{-1}]	q$_S$ [g g^{-1} h^{-1}]	Y$_{X/S}$ [g g^{-1}]
M-0.05	4	0.37	2.23	0.16
	8	0.21	0.86	0.24
	12	0.02	0.06	1.27
	16	0.04	0.10	0.71
	20	0.02	0.12	0.32
	24	0.03	0.11	0.61
	28	0.02	0.12	0.30
	32	0.04	0.19	0.29
M-0.1	4	0.34	2.14	0.15
	8	0.33	0.75	0.34
	12	0.22	0.21	0.88
	16	0.08	0.27	0.63
	20	0.09	0.25	1.06
	24	0.10	0.23	0.67
	28	0.04	0.25	0.39
	32	0.02	0.44	0.02
PM-0.05	4	0.35	1.92	0.18
	8	0.31	0.28	0.79
	12	0.09	0.39	0.34
	16	0.04	0.18	0.76
	20	0.04	0.18	0.90
	24	0.04	0.19	0.74
	28	0.00	0.25	0.02
	32	0.05	0.24	0.31
2PM-0.05	4	0.41	1.91	0.22
	8	0.28	0.17	1.50
	12	0.14	0.26	1.82
	16	0.04	0.18	0.68
	20	0.04	0.18	0.92
	24	0.00	0.19	0.17
	28	0.03	-	-

CHAPTER 5

CONCLUSIONS

In this M.Sc. thesis, it was aimed to compare intracellular glucose isomerase (GI; EC 5.3.1.5) production capacities of two metabolically engineered host cells, *E. coli* and *P. pastoris*. In this context, the study was conducted in two separate parts.

In the first part of the study, intracellular GI producing *P. pastoris* was obtained via transfection. After the selection of the true transformant via PCR, which was called S18 later, expression of *xylA* gene was conducted in a laboratory scale air-filtered shake bioreactor.

❖ Using the defined medium containing methanol as the sole carbon source, A=545 U L^{-1} GI activity was attained. Thereafter, the effect of different sorbitol concentrations was investigated again performing shake flask bioreactor experiments and the highest enzyme activity was obtained as A=792.9 U L^{-1} with addition of C_{S0}=30 g L^{-1} sorbitol initially to the production medium. This approach yielded 1.45 fold increase in enzyme activity.

In the second part of the study GI production capacity of *E. coli* BL21 (DE3) pLysS containing pRSETA::*xylA*$_{int}$ was investigated. With this purpose, a laboratory scale air-filtered shake bioreactor experiment was designed to investigate the effect of different carbon sources, i.e., hydrolyzed and unhydrolyzed molasses, sucrose, glucose + fructose, on the growth of *E. coli* cells. Among all carbon sources, the highest cell growth was attained with hydrolyzed molasses. A laboratory scale shake

bioreactor experiment of *E. coli* BL21 (DE3) pLysS carrying pRSETA::*xylA*$_{int}$ with C_{M0}=30 g L^{-1} hydrolyzed molasses-based medium was conducted and A=2312 U L^{-1} GI activity was obtained. Considering the significant difference (3.17-fold) in activity between *E. coli* and *P. pastoris*, *E. coli* was chosen for bioreactor feeding strategy development experiments with the outcome of laboratory scale shake bioreactor experiment.

Four different feeding strategies including pulse feeding, (NH$_4$)$_2$HPO$_4$ addition to the feed in semi-batch operation, increasing pre-determined specific growth rate, were developed and investigated in pilot scale bioreactor experiments, utilizing molasses-based semi-defined medium initially. Notable findings of the developed feeding strategies can be stated as:

- ❖ (NH$_4$)$_2$HPO$_4$ was added to the feed which was given to the bioreactor with a pre-determined exponential rate in semi-batch operation. The addition of nitrogen and phosphorus sources affected the GI activity in a positive manner.

- ❖ Increasing the pre-determined specific growth rate from μ_0=0.05 to 0.1 h^{-1} caused a consequent increase in feeding rate. However, this additional nutritional source was utilized by the cells for growth instead of GI production. The highest cell concentration was obtained as C_X=17.9 g L^{-1} in this strategy. When compared to the strategy applied with pre-determined specific growth rate of μ_0=0.05 h^{-1}, the maximum cell concentration increased 2.56-fold whereas the lowest GI activity was observed with a 1.8-fold decrease as A=9990.2 U L^{-1}. Moreover, by the end of this strategy, fructose accumulation was observed which was probably due to the high exponential feeding rate.

- ❖ Effect of pulse feeding was investigated in two bioreactor experiments. In the strategy where one molasses pulse was given after batch phase, GI activity increased considerably. Molasses pulse given after batch phase was also favored cell concentration compared to no-pulse condition. Furthermore, it shortened the cultivation time when the maximum GI activity was attained. Nevertheless, feeding second

molasses pulse after the first pulse was not successful at increasing the activity which might be as a result of fructose accumulation within the fermentation medium during semi-batch operation.

❖ The highest volumetric GI activity was attained with one pulse strategy PM-0.05 as A=29050 U L^{-1} at t=26h. 9-7 fold increase in GI activity was obtained compared to the batch production with the developed feeding strategy. The highest specific activity calculated for this strategy was 2303 U g DCW^{-1} (t=26h).

The concentrations of acetic, formic, lactic, malic, succinic and citric acid were determined for all bioreactor experiments.

❖ Among the organic acids, acetic acid was found to be the most predominant. It fluctuated at tolerable limits during the bioreactor experiments which might be the consequence of prevention of nitrogen limitation with feed containing $(NH_4)_2HPO_4$ in semi-batch operation. The highest acetic acid concentration was observed as 4.76 g L^{-1}; the highest total organic acid concentration was observed as 5.4 g L^{-1} with the strategy of M-0.05. Organic and acetic acid concentrations showed an increasing profile for PM-0.05, and high acetic acid concentration was detected especially after the time when the maximum GI activity was attained. The highest acetic acid and organic concentration obtained with the best strategy PM-0.05 were 4.24 g L^{-1} (t=28h) and 5.05 g L^{-1} (t=32h), respectively.

In order to evaluate the bioprocess efficiently, fermentation characteristics such as specific growth rate (μ), specific substrate consumption rate (q_S), overall cell yield on substrate were calculated for different feeding strategies.

❖ After batch phase, lower μ values were observed than pre-determined values of μ=0.05 and 0.1 h^{-1} during semi-batch operation. The highest specific growth rate values are calculated as μ=0.04, 0.1, 0.5 and 0.04 h^{-1} for M-0.05, M-0.1, PM-0.05 and 2PM-0.05 strategies, respectively for semi-batch operation mode. The best strategy in terms of GI activity

PM-0.05 was found to be more successful at maintaining $\mu=0.05$ h^{-1} during semi-batch phase.

❖ q_S values of different feeding strategies showed decreasing profiles with respect to cultivation time. The highest q_S and overall $Y_{X/S}$ values calculated for PM-0.05 are 0.39 g g^{-1} h^{-1} and 0.20 g g^{-1}. Overall $Y_{X/S}$ values were calculated as 0.20, 0.14 and 0.21 g g^{-1} for M-0.05, M-0.1 and 2PM-0.05 strategies, respectively, indicating the similar substrate utilization profile for different strategies. However, lower overall $Y_{X/S}$ values for semi-batch phase were obtained than the $Y_{X/S}$ value (0.32 g g^{-1}) used for the calculation of pre-determined feeding rate for different strategies.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Alkaline Lysis Solution I	Prepared 100 ml batches containing 50 mM glucose, 25 mM Tris-Cl and 100 mM EDTA in distilled water. Autoclaved and stored at 4°C.
Alkaline Lysis Solution II	Prepared 10 ml batches containing 0.2 M NaOH and 1% SDS (w/v) in distilled water. Freshly prepared and used.
Alkaline Lysis Solution III	Prepared 100 ml batches containing 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of dH_2O. Stored at room temperature and transferred to an ice bucket before use.
0.12%(w/v) carbazole	0.12 g carbazole was dissolved in 100ml dH_2O. Freshly prepared and used.
1.5%(w/v) Cysteine HCl	0.75 g cysteine was dissolved in 50ml dH_2O. Freshly prepared and used.
6 x DNA gel-loading buffer	Contained 0.25 % Bromophenol blue, 0.25 % xylenecyanol FF, 40 % sucrose in dH_2O.
GI Activity Buffer	0.4M D-glucose and 10mM $MnCl_2$was dissolvedin 0.02M potassium buffer pH 7.0.

0.1 M potassium phosphate, pH 7.0	3.85 ml of 1M KH_2PO_4, 6.15 ml of 1M K_2HPO_4 was dissolved in dH_2O up to 100 ml. The solution pH was adjusted, then autoclaved and stored at room temperature.
RNaseA stock solution	RNaseA was dissolved at a concentration of 10 mg/ml in 50 mM potassium acetate (pH 5.5) and boiled for 10 minutes. Stored at -20 ˚ C.
70% (v/v) Sulfuric acid	70 ml sulfuric acid was gradually added to 30 ml dH_2O.
10X TE Buffer (pH 8.0)	100 mM Tris-Cl (pH=8.0) and 10 mM EDTA (pH=8.0) dissolved in distilled water. Autoclaved and stored at 4°C. Diluted it 10 times before usage.
5X TBE Buffer	54 g of Tris base, 27.5 g of boric acid and 20 ml of EDTA (0.5 M) were dissolved in 950 ml distilled water. pHwas adjusted to 8.0 and the solution completed up to 1L with distilled water. Diluted5 times before usage.
Antifoam	10% (v/v) antifoam solution was preparedwith distilled water. Autoclaved before use.

APPENDIX B

CALIBRATION OF D-FRUCTOSE CONCENTRATION
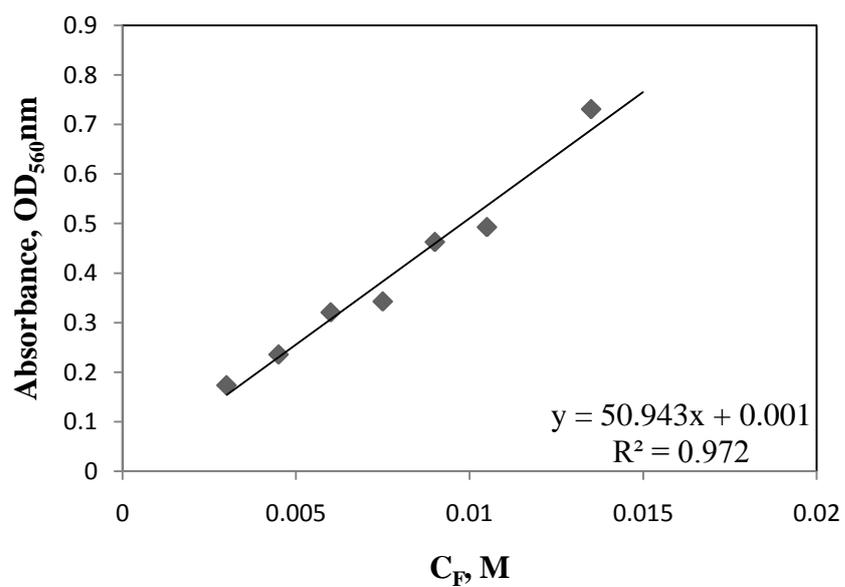
Figure B.1 Standardcalibration curve for D-fructose concentration

According to the equation obtained from the plot and enzyme activity was calculated as:

$$GI\ Activity\ (UL^{-1}) = \frac{C_F\ (\mu M)}{Reaction\ time\ (min)} \qquad B.1$$

$$C_F(\mu M) = \frac{(Absorbance - 0.001)}{50.943} xDilutionFactorx10^6 \qquad\qquad B.2$$

$$GI\ Activity\ (UL^{-1}) = \frac{C_F(\mu M)}{10\ (min)} \qquad\qquad B.3$$

APPENDIX C

CALIBRATION CURVE FOR D-GLUCOSE CONCENTRATION

$y = 0.5716x - 0.0008$
$R^2 = 0.9995$

Figure C.1 Standarcalibration calibration curve for D-gructose concentration

APPENDIX D

CALIBRATION CURVES FOR ORGANIC ACIDS

- **Acetic Acid**

The plot shows Peak Area versus CAA g/L with data points fitted to a line. The equation is $y = 212755x$ and $R^2 = 1$.
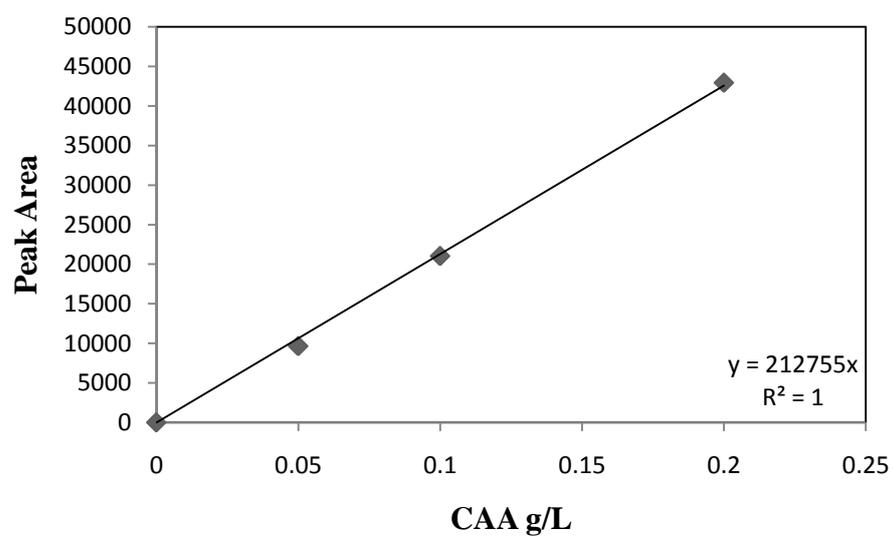
Figure D.1 Standard calibration curve for acetic acid concentration

- **Formic Acid**
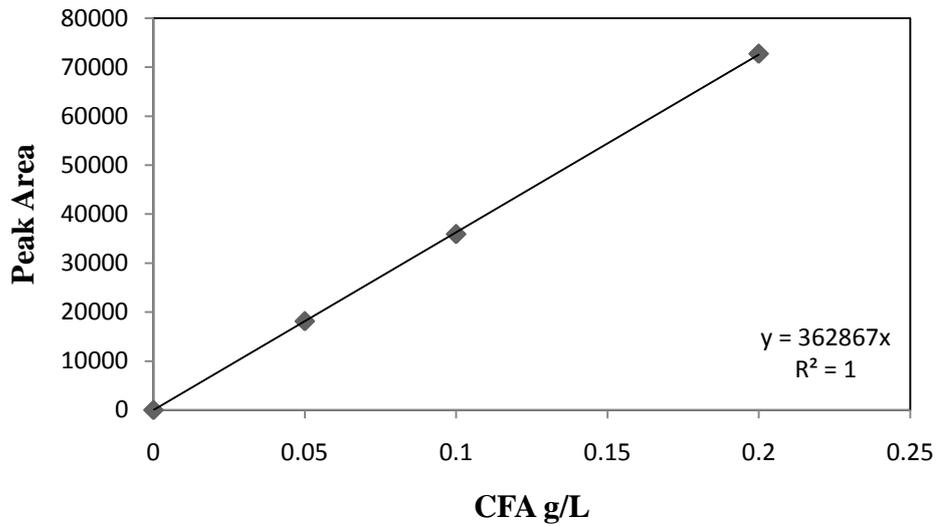
Figure D.2 Standard calibration curve for formic acid concentration

- **Lactic Acid**
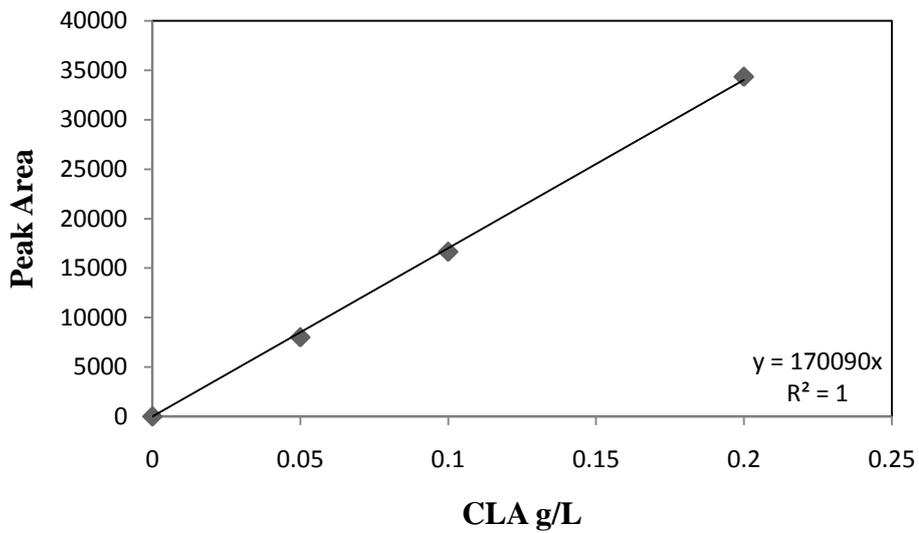
Figure D.3 Standard calibration curve for lactic acid concentration

- **Succinic Acid**
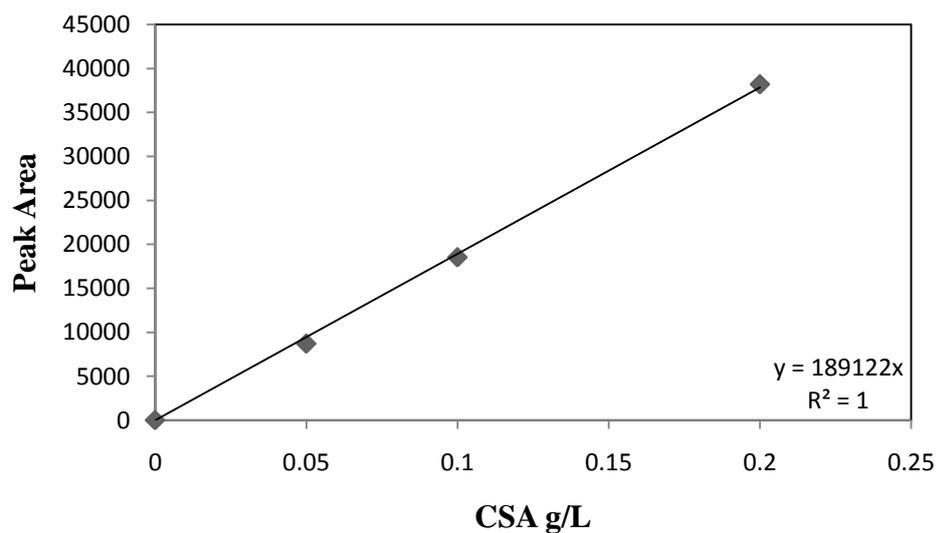
Figure D.4 Standard calibration curve for succinic acid concentration

- **Malic Acid**
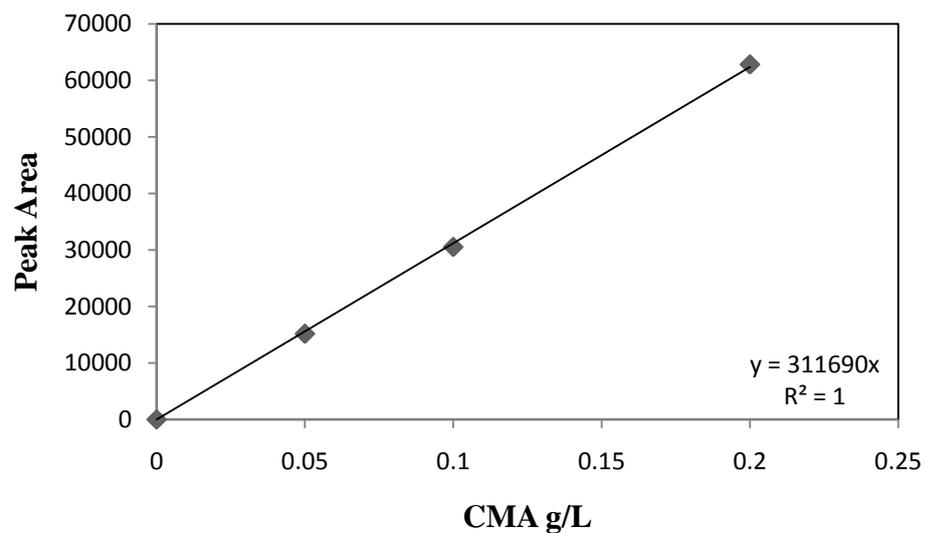
Figure D.5 Standard calibration curve for malic acid concentration

- **Citric Acid**

The chart shows a standard calibration curve with Peak Area on the y-axis (ranging from 0 to 90000) and CCA g/L on the x-axis (ranging from 0 to 0.25). The equation is:

$$y = 408468x$$
$$R^2 = 1$$
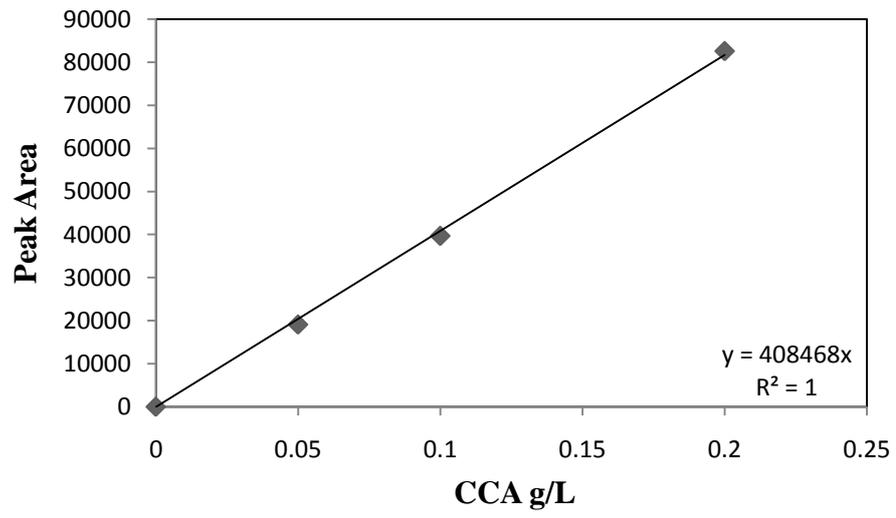
Figure D.6 Standard calibration curve for citric acid concentration